Functional genome analysis of *Alcanivorax borkumensis* strain SK2: alkane metabolism, environmental adaptations and biotechnological potential

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Preface

This thesis is presented to obtain the Ph.D. degree from the Technical University of Braunschweig. The work was performed at the Gesellschaft für Biotechnologische Forschung mbH (GBF) and at the Technical University of Braunschweig, Institute of Microbiology with Dr. Peter Golyshin and Pr.Dr K.Timmis as supervisors. I would like to express my great gratitude to my supervisors Dr. Peter Golyshin and Dr.K.Timmis for the scientific supervision during my Ph.D.

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Summary

A comprehensive functional genomic analysis of the alkane metabolism of the marine oil-degrading bacterium *Alcanivorax borkumensis* SK2 and its concomitant metabolic adaptations is presented. 99 cytoplasmic and membrane-associated proteins of alkane-grown cells were found to be differentially expressed as compared to pyruvate-grown cells as controls. Among these, 46 putative operon structures were identified. While cytoplasmic proteins found to be up-regulated in hexadecane-grown cells mostly represent enzymes of the glyoxylate bypass, of the gluconeogenesis pathway, of the biosynthesis and of the β-oxidation of fatty acids, up-regulated membrane proteins were mostly characteristic for specific responses related to the terminal oxidation and further degradation of alkanes or other functions related to alkane metabolism. Three different enzymatic approaches to the terminal oxidation of alkanes were identified, i.e.enzymes encoded by the previously described alkB1 gene cluster as well as two new gene clusters apparently coding for alternative alkane hydroxylating systems, comprising a p-450 cytochrome-containing monooxygenase and another putative monooxygenase, predicted to be involved in the metabolism of cycloalkanes. The latter two systems of terminal oxidation of alkanes are preceeded by different sigma^{54} and sigma^{70}-dependent promoters, correspondingly and therefore are likely to be differently regulated.

Transposon mutagenesis was used for functional genome analysis of *Alcanivorax* SK2 to identify the genetic basis of environmentally relevant phenotypes, such osmotolerance, UV and low temperature adaptation, and biofilm formation. 48 relevant transposon mutants deficient in any of these environmentally responsive functions were isolated, and the corresponding genes interrupted by the mini-Tn5 element were sequenced using inverse PCR. Analysis of these mutants revealed not only described molecular mechanisms of salt or cold adaptation based on biosynthesis of osmoprotectors, changes of membrane fluidity, or the activation of efflux pumps, but also hinted at novel regulatory phenomena. Tn5 mutants sensitive to UV lacked DNA repair mechanisms, as shown previously, but also the biosynthesis of polymers, and the export of unknown compounds. Biofilm-deficient mutants mostly mapped in genes responsible for signal transduction and regulation, pointing at complex and tightly controlled cellular interactions required for biofilm development. These results open up a door to look now more closely into the role of the corresponding enzymes or regulators that apparently are crucial for adaptation of *Alcanivorax* to its marine environment.

The present study also describes a mutant of *Alcanivorax* strain SK2, which hyper-produces polyhydroxyalkanoate (a raw material for the industrial production of bioplastics) and, more importantly from a biotechnological point of view, excretes the produced polymer into the culture medium. This mutant represents a starting point for the development of a new cellular system for high level production of PHA, since recovery of PHA from culture supernatant fluids circumvents the current major economic and environmental problem of cumbersome and costly extraction of intracellular PHA granules. The localization of the causative mini-Tn5 transposon mutation in a *tesB*-like acyl-CoA thioesterase gene provides new information on the role of *tesB*-like acyl-CoA thioesterases in PHA production, and it may constitute a general strategy to create PHA excreting cellular systems in other PHA producing bacterial species. These findings represent a biotechnological breakthrough that may lead to production of economically-competitive biopolymers.

Abbreviations

BLAST	Basic local alignement search tool
bp	Base pairs
BSA	Bovine serum albumin
CDS	Coding sequence
CV	Cristal violet
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
HPLC	High-performance liquid chromotography
IEF	Isoelectric focusing
IPG	Immobilized pH-gradient
IPTG	Isopropyl-ß-D-thiogalactopyranosid
MALDI MS	Matrix-assisted laser desorption-ionization mass spectrometry
MALDI-TOF	Matrix-assisted laser desorption-ionization time-of-flight
mRNA	Messenger RNA
MS	Mass spectrometry
MW	Molecular weight
kDa	Kilodalton
NCBI	National Center for Biotechnology Information
ND	Not determined
PHA	Polyhydroxyalkanoate
pI	Isoelectric point
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription PCR
SMART	Simple modular architecture research tool
Str	Streptomycin
σ	Sigma
2-DE	Two-dimensional gel electrophoresis
TM	Transmembrane
Tn	Transposon
UV	Ultraviolet
X-Gal	5-Bromo-4-Chloro-3-indoyl-ß-D-galactopyranosid

1. INTRODUCTION

1.1 Bacterial degradation of alkanes and its genetic determinants

Oil spills caused by maritime transport of crude oil and petrol products are still an important source of sea and ocean pollution, especially in main production areas and along major transport routes (Vieites et al., 2004). Of special concern is the danger of a petroleum hydrocarbon spillage in the polar, ice-covered regions due to oil exploration in Arctic offshore areas and a growing interest in using the Northern Sea Route (NSR) as an alternative transportation route for Arctic oil and gas (Gerdes et al., 2005). The impact of accidental oil spills on marine environments is enormous, and it has thus for long attracted scientific attention and instigated studies on the fate of petroleum in marine environment, on the evaluation of the environmental threat of oil spills, and on the development of bioremediation measures to cope with these. Diverse marine microorganisms are able to degrade different compounds of crude oil (Raghukumar et al., 2001; Zinjarde and Pant, 2002; Brakstad and Lodeng, 2005; Harayama et al., 2004), generally with *n*-alkanes being the preferential substrate. Among the various marine oil-degrading microorganisms recently group of marine oligotrophic bacteria was discovered which exclusively use petroleum oil hydrocarbons as source of carbon and energy. These so-called ‘‘hydrocarbonoclastic bacteria’’are normally found in sea water in only low numbers, yet rising dramatically after an oil spill (Golyshin et al., 2003). Thus, within the group of Proteobacteria a number of new genera and families have been recently discovered, comprising the genera *Alcanivorax* (Yakimov et al., 1998), *Cycloclasticus* (Dyksterhouse et al., 1995), *Marinobacter* (Gauthier et al., 1992), *Oleospira* (Yakimov et al., 2003) and *Oleophilus* (Golyshin et al., 2002). However, although quite a number of diverse marine bacteria able to degrade hydrocarbons have now been isolated and described, many unculturable bacteria are likely to remain still undiscovered.

The bacterial ability to degrade hydrocarbons is generally determined by the presence of monooxygenases which catalyse the initial step in the degradation of hydrocarbons. Such monooxygenases are thus widespread in gram-negative oil degrading bacteria, and the encoding genes are usually denoted as alkB genes (Smits et al., 1999). AlkB genes have been shown to ubiquitously encode the enzymes responsible for the primary degradation of alkanes in environments contaminated with fresh crude oil (Sotsky et al.,

1994), and they can therefore be used as biomarkers to assess the oil-degrading potential of a microbial community thriving on oil spills.

The best studied organism with respect to the genetics of alkane degradation is *Pseudomonas oleovorans* GPO1. The *P. putida* GPo1 alkane hydroxylase system consists of three components: alkane hydroxylase (AlkB), rubredoxin (AlkG), and rubredoxin reductase (AlkT). AlkB is a non-heme iron-containing integral membrane protein, which catalyses the hydroxylation reaction (Kok et al., 1989; McKenna and Coon 1970; van Beilen 1992). Rubredoxin transfers electrons from the NADH-dependent flavoprotein rubredoxin reductase (Peterson et al., 1966; Ueda et al., 1972) to AlkB. The molecular genetics of the alkB hydroxylase system has been reviewed by van Beilen et al. (van Beilen et al., 2001; van Beilen et al., 1994). Not only plays the *P. putida* GPo1 alkane hydroxylase an important role in the initial attack of alkanes, it has also been shown to be a versatile biocatalist attracting quite some industrial interest, as it is involved in a wide range of biological processes, i.e. in the hydroxylation of linear and branched aliphatic, alicyclic, and alkylaromatic compounds; in the oxidation of terminal alcohols to the corresponding aldehydes; demethylation of branched methyl ethers; sulfoxidation of thioethers; and in the epoxidation of terminal olefins and allyl alcohol derivatives (McKenna and Coon, 1970; van Beilen et al., 1994; Katopodis et al., 1988; Katopodis et al., 1984; May et al., 1972; 1986; Fu et al., 1991).
However, alternative alkane hydroxylating systems other than alkB, have also been described. For example, it was shown that some oil-degrading bacteria contain an alkane hydroxylase system belonging to the cytochrome p-450 family, as it was proposed for *Rhodococcus rhodochrous* ATCC 19067 and *Acinetobacter calcoaceticus* EB104 (Cardini and Jurtshuk, 1968; Müller et al., 1989).

1.2 *Alcanivorax* strain SK2 as an oligotrophic, oil-degrading marine bacterium

Alcanivorax borkumensis strain SK2 is a cosmopolitan marine bacterium, belonging to the gamma-proteobacteria, and a prime representative of the hydrocarbonoclastic group, with a specialized metabolism adapted to the degradation of petroleum oil hydrocarbons, but rendering it unable to utilize many other common sources of carbon and energy (Yakimov et al., 1998). *Alcanivorax* has now been detected in samples from different geographical locations, such as the Atlantic Ocean, Mediterranean Sea, North Sea, Sea of Japan, South China Sea, and the Antarctic (Golyshin et al., 2003). It

degrades a wide range of hydrocarbons, it is usually the most abundant member of microbial communities that develop following an oil spill at sea, and it is thus assumed to be one of the globally most important microbes involved in removing oil from marine environments (for review see Golyshin et al., 2003). The list of sites where it has been isolated is constantly expanding (Röling et al. 2004; Cubitto and Cabezali 2001).

Among the variety of other species of hydrocarbonoclastic bacteria, *A. borkumensis* features a unique physiology marked by distinct oligotrophy (Golyshin et al., 2003). As an adaptation to its marine environment, which is characterized by very heterogeneous distributions of nutrients, oligotrophic bacteria such as *Alcanivorax* have evolved remarkable nutrients scavenging capabilities which allow these bacteria to grow even at very low external nutrient concentrations. Usually representing an only minor fraction of the community in non-polluted sea waters, their numbers drastically increase after an oil spill, thus rendering it the dominant member of the respective microbial community (Harayama et al., 1999; Kasai et al., 2001; Kasai et al., 2002; Syutsubo et al., 2001). In such conditions, when nutrients suddenly become abundant, oligotrophic bacteria and notably *Alcanivorax borkumensis* may store this excess of carbon in form of intracellular granula consisting of polyhydroxyalkanoate polymers. Such storage capabilities are presumably widespread among oligotrophic marine bacteria, signifying an adaptation mechanism to survive between carbon surplus situations like e.g. oil pollution events.

Another interesting feature of metabolism of *Alcanivorax* is its restricted substrate profile marked by a strong preference for alkanes. It has recently been shown that *Alcanivorax borkumensis* posesses more than only one system for the terminal oxidation of alkanes, with at least two *alkB*-like genes, namely *alkB1* and *alkB2* encoding alkane hydroxylases (Hara et al. 2004; van Beilen et al. 2004). Transcriptional expression studies on *A. borkumensis* strain AP1 have revealed that expression of both alkB1 and alkB2 was induced by C_{14} alkanes in *A. borkumensis* AP1 (van Beilen et al., 2004). However, out of these two genes only the *AlkB1* gene was shown to be essential for the degradation of C_6 alkanes in *A. borkumenis* SK2, as shown by knockout mutagenesis of the corresponding genes (Hara et al., 2004). As for growth on alkanes ranging from C_8 to C_{16}, both individual single knockouts (alkB1 and alkB2), and a double mutant defective in both alkB1 and alkB2 genes, did not exhibit marked growth deficiencies as

compared to the wild type. Therefore, it was postulated that genes other than *alkB1* and *alkB2* must be responsible for the degradation of such alkanes.

Because of its global importance, unusual physiology, and its potential for biotechnological applications displaying multiple systems for alkane degradation, *Alcanivorax borkumensis* has recently been sequenced, and the functional analysis of the sequenced genome is being initiated with the thesis presented here. The main goal of conducting a functional genome analysis of this particular microorganism was to understand the molecular basis of its ability to degrade alkanes and to understand its responses to environmental stresses at the genomic level, making it the most successful and consequently dominant species of marine communities of oil-degrading bacteria.

1.3 Functional genome analysis and its methods

The completion of hundreds of bacterial whole-genome sequences has generated a large amount of sequence information. Such large amount of *in silico* information can be first approached by bioinformatics, whose role is to predict and assess the metabolic potential and specificity of the physiological features of the sequenced organism. The information drawn from the annotated genome (genome with assignments of putative ORFs) provides important baseline knowledge for more in-depth functional genome studies, for instance to determine which genes a given organism utilizes for its adaptation to certain environmental conditions allowing for its survival and growth. Functional genome analysis or functional genomics operates with the whole genome and denotes a genome-wide screening for genes differentially expressed under conditions of interest. There is number of functional genomic methods, which on their own or in combination with each other provide a comprehensive view of the bacterial metabolism under differential conditions.

The classical method of genome-wide analyses is genome-wide random transposon mutagenesis, based on the use of transposons or mobile genetic elements which are randomly inserted into different regions of the chromosome thereby creating knockout mutations (Hayes, 2003). The sites of the transposon insertions can be identified by sequencing the regions flanking the transposon, thus identifying the gene whose interruption caused loss of function. Genome-scale analyses based on the use of transposon mutagenesis has been successfully performed in a number of studies

(Gehring et al., 2000; Hutchison et al., 1999; Fouts et al., 2002). Being one of the most powerful methods of functional analysis, it still has its limitations. One of the disadvantages of classical transposon studies is its inability to select for lethal mutations. A second drawback is its inherent inability to study redundant functions. For example, if there is more than one alternative route for one metabolic transformation, transposon mutation of any one of them would not be resulted in the loss of function, thus allowing for linking gene and function.

Another method of functional genome analysis is the proteomic approach which requires the use of two-dimensional gel electrophoresis to identify all proteins expressed under the conditions of interest. Proteomic analyses were first conducted already in 1975 (Klose, 1975). A renewed interest in this technology is caused by several recent advances. Among them are the availability of public genome and protein databases, the development of high-sensitivity, easy-to-use mass spectrometers, improved two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), and computer programs for analysis of 2D-PAGE gel images (Resing 2002). In contrast to transposon mutagenesis, which basically is based on negative selection of specific phenotypes, proteomics allows overcoming the aforementioned disadvantages associated with the use of transposon mutagenesis, i.e. allowing to study expression of redundant protein functions, as well as to identify proteins, inactivation of which would be lethal for the cell. This technique is also an essential complement to transcriptome analysis (see below) because in principle it looks at the proteins themselves, the actual functional entities of a cell, including their potential post-translational modifications, which cannot be predicted by mRNA expression analysis (Sauer, 2003). Having the reputation of being the most powerful tool of functional genomics, this technique still has quite some technical limitations, such as low resolution and detection limits (Sauer, 2003). Another drawback of this technique is the difficulty to properly resolve hydrophobic membrane proteins on 2-DE gel maps.

DNA microarray technology allows for parallel nucleic acid hybridizations for a large number of immobilized oligonucleotides or larger DNA fragments on a small surface area (Service 1998), forming the basis of identifying large members of mRNA transcripts at the same time, an experimental approach for which the general term transcriptomics has been coined. Since the invention of microarray technology in 1995,

it has been extensively used for monitoring the genome-wide expression of genes in a number of prokaryotes (Schoolnik 2002; Shoemaker and Linsley 2002; Richmond et al., 1999). Not only can transcriptomics be used to look at the expression of the whole genome, but it can also successfully elucidate complex transcriptional regulatory networks, when looking at mutants deficient in the corresponding transcriptional regulators (Mader et al., 2002). Other areas of application of this technology lie in identifying bacterial species (Gingeras et al., 1998; Guschin et al., 1997; Wilson et al., 2002) and monitoring the composition of microbial populations (Small et al., 2001; Koizumi et al., 2002). The application of DNA microarrays specifically in environmental microbiology may significantly improve the understanding of complex microbial communities, which are typically composed of many microbial species (Urakawa et al., 2003). However, a central challenge to the application of DNA microarrays in environmental microbiology is to achieve a high degree of specificity needed to resolve complex microbial populations, including discriminating between target and nontarget bacterial groups that differ by only a single nucleotide (Guschin et al., 1997). Another major bottleneck of the microarray technology is the cumbersome processing and analysis of transcriptome data and its precarious use for purposes other than the quantification of changes in gene expression levels (Dharmadi and Gonzalez, 2004). For this reason, it may often be desirable to use DNA microarray technologies in conjunction with other genome-wide methodological approaches, e.g. proteomics, metabolomics, etc., which will provide a better assessment of the genotype-phenotype relationships in bacteria.

1.4 Environmental adaptation of marine bacteria

1.4.1 Resistance to ultraviolet radiation

Organisms living in a marine environment are exposed to constant pressure of a combination of different environmental factors, most importantly ultraviolet radiation, salinity and low temperatures. Solar ultraviolet radiation produces continuous stress on marine communities, causes cellular damages that instantly need to be repaired. Moreover, progressing deterioration of the earth's stratospheric ozone layer leads to an increased impact of ultraviolet radiation (UV) also on marine bacteria. Solar UV wavelengths of biological importance mainly comprise UVA (320 to 400 nm) and UVB (290 to 320 nm). Besides its implication in causing damage to proteins and membranes, UVA indirectly damages in particular DNA by generating reactive oxygen compounds

(e.g., H_2O_2, O_2^-, etc.) through photooxidation of O_2 entailing single-strand breaks in DNA (World Health Organization, 1994). UVB, on the other hand, is directly absorbed by DNA as it affects nucleotides by creating cyclobutane pyrimidine dimers and photoproducts (Friedberg et al., 1995). UVC (100 to 290 nm) constitute only a small fraction of the UV radiation, but it is the most energetic. Although normally completely blocked by the ozone layer, it has now been studied more extensively because of its germicidal effects (Friedberg et al., 1995) due its recent penetration through the growing leaks in the earth's ozone layer.

In response to DNA-damaging agents such as UV radiation, bacteria evolved many diverse ways to eliminate or repair the damaged DNA, the so-called photoreactivation, the SOS response system, nucleotide excision repair mechanisms, and methyl-directed mismatch repair. Apart from the genes encoding enzymes that are directly involved in the DNA metabolism, there are other genes shown to be relevant for UV tolerance, such as genes involved in cell division and various specific metabolic pathways which are apparently unrelated to DNA repair (Duwat et al 1997).

Photoreactivation is one of the most important and most frequently encountered repair mechanisms present in a variety of organisms (Hader and Sinha 2005). Kelner and Dulbecco were the first to report already in the 1940s and 1950s that UV-induced damage in bacteria and phage can be reversed by illumination with visible light, a phenomenon that later was named photoreactivation (Cleaver 2003). Photoreactivation in bacteria relies on the activity of a single enzyme called photolyase, which binds to DNA photoproducts and, in the presence of light (300 to 500 nm), reverses the dimers to their component monomers (Kim and Sancar, 1993). Photolyases are widespread among bacteria, archaea, and eukaryotes (Yasui and Eker, 1998). However, the role of photoreactivation in DNA repair should not be over-estimated, since most photolyases only act on pyrimidine dimers and can not reverse other DNA lesions (Yasui and Elker, 1998). Moreover, photoreactivation was not found to contribute to UV adaptation in *Haemophilus influenzae, Streptococcus pneumoniae, Bacillus subtilis, Neisseria gonorrheae*, nor for the remarkable degree of UV resistance characteristic for *Deinococcus radiodurans*, as this mechanism is reported to be entirely absent in these bacteria (Campbell and Yasbin 1979; Harm 1976).

Another mechanism contributing to cellular UV tolerance is nucleotide excision repair, which cleaves DNA on both sides of the damaged nucleotide, and then fills in and seals the single-stranded gap after removal of the damage-containing oligonucleotide (Sancar and Rupp 1983). This mechanism is widely assumed to be the major route for removal of nucleotide damages from the DNA. (A)BC excinuclease is the primary nuclease activity mediating nucleotide excision repair in *E.coli*. Six genes*, uvrA, uvrB, uvrC, uvrD, polA,* and *lig*, are required to encode this enzymatic process in *E. coli*. In contrast to photoreactivation, nucleotide excision repair, can actively repair both cyclobutane pyrimidine dimers and so-called 6-4 photoproducts, being the two most common types of lesions caused by UVB and UVC irradiation (Pfeifer 1997). Nucleotide excision repair was also shown to account for the remarkably high UV resistance of *Deinococcus radiodurans*, a species that was first isolated from X-ray sterilized canned meat, that despite this treatment was found to have undergone spoilage (Anderson et al 1956). However, differently from *E.coli*, UV resistance in this bacterium is attributed to the presence of two other UvrABC-like excision repair systems, acting simultaneously (Minton 1994).

Many of the genes involved in DNA damage repair are inducible through the so-called SOS response system (Little and Mount, 1982). It was Evelyn Witkin, who first hypothesized in 1967 that bacterial cell division is controlled by a repressor which is inactivated by a complex process that starts off from replication-blocking lesions in the DNA (Bridges 2005). This suggestion later gave a rise to the SOS response hypothesis: DNA damage as signalled by the presence of single-stranded DNA (Sassanfar and Roberts, 1990), is sensed by the highly conserved RecA recombinase protein, which facilitates inactivation of a transcriptional repressor, LexA. Inactivation of LexA in turn causes induction of genes belonging to the LexA regulon, many of which are involved in DNA repair and cell's survival after DNA damage (Au et al., 2005). Approximately 40 genes have been reported to belong to the SOS regulon of *E. coli* (Courcelle et al., 2001), and most of these genes exhibit in their promoter/operator regions a 20-bp LexA-binding consensus motif, the "SOS box" (Walker et al., 1984).

One further major bacterial pathway to deal with UV-induced DNA damages is methyl-directed mismatch repair, mostly implicated in the processing of UV photoproducts in DNA (Feng et al., 1991). This mechanism of mismatch correction, postulated in 1976

by Wagner and Meselson, is directed to newly synthesized DNA and functions by eliminating replication errors (Wagner and Meselson, 1976). DNA methylation was also suggested to provide the molecular basis for allowing for the stand discrimination in this type of repair, with newly synthesized DNA transiently being methylated to a lesser degree than the parental template strand. This type of repair requires function of the MutH, MutS, MutL, and UvrD proteins and of a specific DNA-methylase Dam (Lahue et al., 1987). This mismatch-repair system decreases DNA replication error rates by 100- to 1000-fold, by way of recognition and correction of base/base and of (insertion/deletion)-loopout mismatches that escape proofreading by DNA polymerase (Kornberg and Baker, 1992).

A recent comparison by Ghosal et al. (2005) of the UV-induced responses in naturally UV-sensitive (*Shewanella oneidensis*) and UV-resistant bacteria (*Deinococcus radiodurans*) has lead to the interesting hypothesis that UV adaptation in these bacteria is linked to the intracellular accumulation of different metals: with *Deinococcus* accumulating manganese and Shewanella accumulating iron. UV-induced cell death in the case of *Shewanella* might be induced by the release of Fe(II) from proteins during irradiation, leading to Fe(II)-dependent oxidative stress, causing additional cellular damage. In contrast, Mn(II) ions that are intracellularly accumulated in *Deinococcus* are considered to serve as antioxidants by that reinforce enzymic systems which defend against oxidative stress.

1.4.2 Osmoadaptation

Marine bacteria must be able to cope with significant changes in extracellular osmolarity, because of perturbation of salinity and resulting osmolarity gradients forming in their respective marine habitats. To respond to osmotic stress, bacteria have developed specific mechanisms aiming at the immediate restoration of the osmotic balance. A prime function in the bacterial adaptation to osmotic upshifts is borne by the action of different membrane transporters (Calamita et al., 1995; Wood, 1999). Acting as efflux pumps, such transporters mediate the energy-dependent export of ions against their gradient, with the required energy drawn from coupling to exergonic enzymatic metabolic processes or respiratory steps, or by utilizing another excisting ion gradient, e.g. by catalyzing the efflux of Na^+ in exchange for imported H^+ (Dimroth, 1997; Padan and Krulwich, 2000). In *Escherichia coli*, the immediate response to osmotic stress

exerted by high external salinity is through K^+ uptake via the so-called Kdp and Trk systems (Bakker, 1992; Schlösser et al., 1995). The main ion causing osmotic stress and having toxic effects on the cells is Na^+. Therefore, the intracellular concentration of Na^+ is kept at a non-toxic level via the functioning of Na^+ efflux systems in many bacteria. Another more general mechanism recognized to be important for conferring osmotolerance is the uptake or the *de novo* biosynthesis of compatible solutes. Compatible solutes are zwitterions, which therefore carry no charge. In contrast to ion accumulation, e.g. K^+ uptake in case of *E.coli*, intracellular accumulation of such uncharged molecules restores osmotic balance of cells exposed to high external osmotic pressure, without causing damaging effects on proteins and other cellular constituents. A range of such compatible solutes is synthesized, imported, or exported by a number of different bacteria. Common compatible solutes include amino acids such as glutamate and proline, amino acid derivatives such as glycine betaine and ectoine (1,4,5,6-tetrahydro-2-methyl pyrimidine-4-carboxylate), and sugars such as trehalose and mannitol (Ciulla and Roberts, 1999; Dinnbier et al., 1988; Kets et al., 1996; Talibart et al., 1994; Welsh et al., 1991).

1.4.3 Resistance to low temperature

It is generally recognized that cold shock proteins play an important role in adaptation of different bacteria to low temperatures. The cold shock proteins are small peptides that share a conserved domain, called the cold shock domain (CSD) that is important for nucleic acid binding and thereby performing a regulatory function in adaptation to cold shock. Many bacteria contain multiple cold shock proteins (Lang and Marques, 2004; Phadtare and Inouye, 2004; Thieringer *et al*. 1998; Rabus et al., 2004). In addition to the known cold shock-inducible genes, a DNA microarray profiling of *E. coli* in response to cold shock showed new genes such as the flagellar operon, those encoding proteins involved in sugar transport and metabolism, and remarkably, genes encoding certain heat shock proteins also induced by cold shock (Phadtare and Inouye, 2004). Cold shock of a hyperthermophilic archaeon *Pyrococcus furiosus* resulted in multiple responses, which included proteins involved in translation, solute transport, amino acid biosynthesis, and tungsten and intermediary carbon metabolism, as well as numerous conserved-hypothetical and/or membrane proteins (Weinberg et al., 2005). The few other genes that have been found to be relevant include *rbfA* (encoding ribosome-

associated factor) and *csdA* (encoding DEAD-box helicase), *pnp* (encoding the polynucleotide phosphorylase that is involved in RNA degradation) (Luttinger *et al*. 1996), *hns* (encoding DNA-binding histone-like nucleoid protein) (Dersch *et al*. 1994), and *bipA* (encoding ribosome-associated GTPase) (Pfennig and Flower 2001).

Relatively little is known about the molecular mechanisms of adaptation of psychrophilic bacteria to low-temperature growth. In the psychrotrophic species *Listeria monocytogenes*, a deficiency to produce the oligopeptide-binding protein, OppA, resulted in cold sensitivity of a respective mutant (Borezee et al. 2000). A cold-sensitive mutant of *Ps. syringae*, a highly cold-adapted Antarctic bacterium, bearing a transposon insertion in its recD gene, accumulated DNA fragments during growth at 4°, indicating that the RecD protein of this psychrotroph might play a vital role in DNA repair, especially during growth at low temperature (Regha et al. 2005).

1.4.4 Bacterial biofilm formation

Studies of ecosystems and the physiological status of microbial communities have long been focused on liquid bacterial cultures. However, these studies do not address the state of microorganisms living in more complex environments such as biofilms. A biofilm is definied as a dense aggregation of microbial cells bound together by a slimy extracellular matrix of polysaccharide and protein (O'Toole and Stewart, 2005). Bacterial biofilm formation has been described as a developmental process consisting of three stages: the planctonic stage, the monolayer stage, and the biofilm stage (O'Toole et al., 2000). Bacteria in the planktonic stage are attached to neither each other, nor to any alien surface; bacteria in the monolayer stage are attached to surfaces as single cells; and bacteria in the biofilm stage are attached to surfaces as cellular aggregates.

Genes required for biofilm formation have mostly been studied in pathogenic bacteria, due to the specific interest from clinical research in the observed resistances of biofilms of pathogenic bacteria towards antibiotics (Davies et al., 1993; Garcia et al., 2004; Wen and Burne, 2002). The subsets of genes that have been implicated in biofilm formation in different pathogenic bacteria are immensely diverse, reflecting the high degree of complexicity of the development of the bioflms. A number of studies on biofilms were carried out using DNA microarrays, revealing a global change of gene expression during growth of cells in biofilms as compared to cellular growth in the planctonic state.

In *Ps. aeruginosa* the well-described phenomenon of quorum sensing has been shown to be involved in the formation of biofilms, which contributes to this bacterium's ability to initiate infection and persist in a host (Davies et al., 1993). Among other genes, found to be up-expressed in *Ps. aeruginosa* cells growing in biofilms were genes responsible for motility, attachment, translation, metabolism, transport and various regulatory functions (Whiteley et al., 2001). Among the genes that in *E.coli* show increased expression during growth in biofilms are genes involved in adhesion and autoregulation, several genes encoding important outer membrane proteins such as OmpC, OmpF, OmpT, genes needed in particular under oxygen- and nutrient-limiting conditions, as well as genes associated with heavy-metal resistance (Schembri et al., 2003).

There is also a number of recent studies, that pointed at cellular secondary messengers playing a key role in bacterial biofilm formation, in particular cyclic-di-GMP (Jenal 2004; Hoffman et al., 2005; Kirillina et al., 2004). Cyclic-di-GMP consists of two molecules of GTP. Proteins that catalyse the synthesis of cyclic-di-GMP have a conserved core of a GGDEF amino acid motif, and are refered to as diguanylate cyclases. Counteracting enzymes that degrade this metabolite, called phosphodiesterases, are characterized by the presence of an EAL tri-aminoacid motif (Ross et al., 1991). Thus, in different bacterial species a number of genes containing either a GGDEF domain or an EAL domain, or both of these have been found to be involved in different aspects of biofilm formation (Kirillina et al., 2004; Hickman et al., 2005; Karatan et al., 2005).

1.5 State of the art and objectives of the project

Alcanivorax borkumensis features a unique physiology marked by distinct oligotrophy and a very restricted substrate profile. Because of its global importance, unusual physiology, and its potential for biotechnological applications, the organism has recently been sequenced, and the functional analysis of the sequenced genome has now been initiated with the present study. The main goal of the functional genome analysis of this particular microorganism was to understand the molecular basis for its remarkable ability to degrade alkanes, as well as a basic genomic understanding of the cellular responses to major environmental stress factors, ensuring its successful thriving as a dominant species in marine oil-degrading bacterial communities.

The specific objectives of the project included the following:
1. To identify via functional genome analysis methods the genes involved in alkane metabolism of this marine oil-degrading bacterium *Alcanivorax borkumensis* SK2. Thus, mini-Tn5 transposon mutagenesis was employed to generate and select for mutants deficient in growth on alkanes. Secondly, as a complementary approach two-dimensional gel electrophoresis was employed to positively screen for potentially up-regulated monooxygenases and other proteins indicative for alkane degradation, in both the membrane and the cytoplasmic fractions of *A. borkumensis.*

2. To gain new insights into the genomic basis of Alcanivorax borkumensis' responses to environmental stress factors, mini-Tn5 transposon mutagenesis was employed to screen for UV, salt and low temperature sensitive as well as biofilm deficient phenotypes, and selected mutants were sequenced to identify the genes interrupted by Tn5 transposons, which are relevant for the respectively observed mutant phenotypes.

2. MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

A. borkumensis SK2 (DSM No. 11573) was used as the wild type for all experiments. Its mini-Tn5 mutants were generated by standard procedures using mini-Tn5 Str/Sp element (de Lorenzo et al., 1990). The SK2 wild type and the mini-Tn5 mutants were grown at 30 ºC in modified ONR7a medium containing 0.27 g/l of NH_4Cl and either 1.5% (w/v) octadecane or 2% (w/v) pyruvate as carbon sources (Yakimov et al., 1998). *E. coli* strains used for the mini-Tn5 transposon mutagenesis were *E. coli* CC118 (λ pir) and 101HB λ pir. *E. coli* strains used for cloning and expression of the tesB-like gene were DH5α (Invitrogen; Carlsbad, CA, USA) and Rosetta Blue DE3 (Novagen; Madison, Wisconsin, USA). The E.coli strains were grown at 37 °C in Luria-Bertani medium with kanamycin (50μg/ml), or streptomycin (50μg/ml) and/or chloramphenicol (34 μg/ml), where appropriate.

2.2 Biofilm formation assay.

2.2.1 Screening for mutants defective in biofilm formation

In order to screen for the transposon mutants defective in biofilm formation, a procedure described by O'Toole and Kolter (1998) was used with some modifications. This assay is based on the ability of bacteria to form biofilms on polyvinylchloride plastic (PVC), a material used to make catheter lines. Biofilm formation was assayed by the ability of cells to adhere to the wells of 96-well microtitre dishes made of PVC as following. The ONR7a medium (100 μl/well) was inoculated in 96-well microtitre plates using replicator device. After inoculation, plates were incubated at 30 degrees for 72 hours, then 25 μl of a 1% solution of cristal violet (CV) was added to each well (this dye stains the cells but not the PVC), the plates were incubated at room temperature for approximately 15 min, rinsed thoroughly and repeatedly with water and scored for the formation of a biofilm as described in the section 2.2.2.

2.2.2 Quantification of biofilm formation

Biofilm formation was quantified by the addition of 200μl of 95% ethanol to each CV-stained microtitre dish well, of which 125 μl was transferred to a new polystyrene microtitre plate, and the adsorbance was determined with a plate reader at 600 nm (series 700 microplate reader; Cambridge Technology).

2.3 PHA isolation and composition analysis.

To analyse the PHA produced and released into the medium by the wild type *A. borkumensis* SK2 and by the C9 mutant strain, the bacteria were cultured in ONR7a medium containing either 2% of pyruvate or 1.5% octadecane as carbon sources on a rotary shaker (100 rpm) at 30^{o} C until late stationary phase of growth. The SK2 cells were harvested by centrifugation (60 min x 12,000 rpm) and the cell pellet and supernatant were collected, recovered and lyophilised. As for the C9 mutant, a total culture was liophilised and used for the chemical analysis. To quantify PHA, aliquots of the cell, supernatant or culture powders were rinsed with ice-cold water and dried overnight at 80^{o}C under vacuum. The freeze-dried sample (cells or supernatant) from each culture were accurately weighed. PHAs were extracted from the lyophilized samples by Soxhlet extraction with chloroform for 16 h (Cromwick et al., 1996). The chloroform solutions were filtered to remove any cellular debri, concentrated by rotary evaporation to 1 mL, and added dropwise to cold (-20ºC) methanol (1:10 v/v) to precipitate PHA polymer. After the polymer precipitate had settled, the supernatant was decanted and the precipitate washed with methanol and dried in vacuo. The dried polymer was weighed, and the yield of polymer as a percentage of the cell dry weight or culture volume was calculated.

To determine the PHA composition, 2 mg of purified PHA were incubated with of a mixture of chloroform:methanol:sulphuric acid (1:0.85:0.15ml) for 2 h at 100^{o}C to degrade PHA by methanolysis to its constituent ß-hydroxycarboxylic acid methyl esters (FAME). Distilled water (0.5 ml) was then added, the tube was shaken for 1 min and then the phases were allowed to separate. The organic phase was transferred into a vial and the FAMEs were analysed with a gas chromotograph-mass spectrometer (GC/MS, model Varian 3400CX, Varian Chromatography Systems, Sugar Land, TX, and VG Autospec spectrometer), equipped with a 30 m x 0.25 mm HP-5 (5% diphenyl and 95% dimethylpolysiloxane) fused silica capillary column (flow rate 1 ml/min; sample input temperature to 230^{o}C at a rate of 8^{o}C/min; interface temperature 250^{o}C; ion source temperature 175^{o}C; electron impact mode 70 eV; scanning from 45 to 450 amu at 0.5 s/scan). The degree of purity of the PHA samples used for analysis was about 99.5%; no trace amounts of proteins, carbohydrates or lipids were detected. The samples were analysed by gel permeation chromatography in a HPLC system with a Spectra-Physics pump and an Aminex HPX-87H column (Bio-Rad, Hercules, CA., USA) under the

following conditions: column temperature, 50°C; gradient, isocratic; mobile phase, 5mM sulfuric acid; flow rate, 0.5 ml/min; detector, light scattering.

2.4 Electron microscopy

In order to detect PHA granula in cells of SK2 and C9 strains, the cells were cultivated in ONR7a containing either 0.15% (w/v), or 1.5% (w/v) octadecane, or either 0.2% (w/v) or 2% (w/v) pyruvate and 0.27 g/l NH_4Cl and were harvested at the stationary phase of growth. Embedding and ultrathin sections were done as it is described by Yakimov et al. (1998). In order to characterise biofilms produced by SK2 wild type strain and C9 mutant, cells were grown on Permanox ® slides (Nalge Nunc) under the same conditions, in the presence of 1.5% of octadecane and scanning electron microscopy was performed as described by Lünsdorf et al. (2001).

2.5 Construction of a mini-Tn5 transposon library of *A. borkumensis* SK2

Transposon mutagenesis was based on the mini-Tn5 Str/Sp element constructed by de Lorenzo et al. (1990). *A. borkumensis SK2* was grown at 30° on ONR7a medium until the stationary phase of growth and cells were centrifuged at 4000 rpm at 4°C. The donor strain *E. coli* CC118 (λ pir) and helper (101HB λ pir) cultures of *E. coli* were grown overnight at 37° on LB medium with either streptomycin or chloramphenicol respectively, washed with fresh LB and centrifuged at 4000 rpm at 4°C. The pellets of *A. borkumensis* and *E. coli* donor and helper strains were mixed in proportion 4:1:1 and placed on a membrane filter on a plate with LB agar and salts ($Na_2HPO_4x\ 2H_2O$ – 0.45; $NaNO_3$ – 2.5; NaCl – 11.5; KCl – 0.38; $CaCl_2x2H_2O$ – 0.7 g/l) and 2% pyruvate as carbon and energy source. The plate was incubated for 24 hours at 30°C. The cells were then washed with 10mM $MgSO_4$ and transconjugants were selected on ONR7a with 0.5% pyruvate and 0.5% acetate as carbon sources and nalidixic acid (10 μg/ml) and streptomycin as antibiotics as required.

2.6 Inverse PCR

The mini-Tn5 insertion sites of the selected mini-Tn5 mutants were determined by inverse PCR as described previously (Ochman et al., 1988). In short, total DNA of the mutant was isolated and digested with *ClaI*, which does not cut within the mini-Tn5 element. The resulting DNA fragments were circularized with DNA ligase and the flanking regions of the inserted mini-Tn5 were amplified with two primers

corresponding to the OTR End (GGC CGC ACT TGT GTA TAA GAG TCA G) and the 1TR End (GCG GCC AGA TCT GAT CAA GAG ACA G), respectively. The conditions for the PCR were: 94^{o}C 1.5 min; 48^{o}C 1 min; 70^{o}C 4 min, 30 cycles. The PCR products were gel-purified and used for automatic DNA sequencing with BigDye terminators on an ABI Prism 377 sequencer (AP Biosystems). To determine the precise site of transposon insertion, additional primers have been designed to read the flanking regions of the disrupted gene, i.e. 1086 (TTA CTG GCT TCG CAG GAA TGG) and intSM160 (CTT GGC ACC CAG CAT GCG CGA GCA GG).

2.7 Reverse transcription PCR

In order to determine whether the two genes (ABO_1111 and ABO_1112) constituted an operon, reverse transcription PCR (RT-PCR) was performed. Using a Fast Blue RNA isolation kit (Qbiogene) total RNA was extracted from 10 ml of culture of *A. borkumensis* SK2 grown to early stationary phase on either 2% pyruvate or 1.5% octadecane. Primers used for RT-PCR were Oligo I (TAT GGT CAA AGT CAG GCG GTG) and Oligo II (CAC ATC CAA GCG CAA AGA CTG) (specific for a 311-bp region spanning the ABO_1111 and ABO_1112).

RT-PCR was also performed with RNA isolated from both *A.borkumensis* SK2 wild type and a *tesB*::Tn5 mutant to determine whether the generated mutation had a polar effect on the downstream gene. It was carried out using the primers mentioned above. To avoid DNA contamination, template RNA was treated with DNase I (Invitrogen) prior to RT-PCR. RT-PCR was performed using the SuperScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen), according to the supplier's instruction. The reaction mixture (10 µl) contained 2 µl of template RNA (DNase I treated), 1µl of a 10 mM dNTP mix, 1 µl of 2µM primer Oligo II, and 6 µl of DEPS-treated water. The samples were incubated at 65^{o}C for 5 min, then placed on ice and 9 µl of the reaction mixture containing 2µl 10xRT buffer, 4 µl of 25 mM MgCl2, 2 µl of 0.1M DTT, and 1µl of RNaseOUT Recombinant RNase inhibitor were added. The mixture was incubated at 42^{o}C for 2 min and 1µl (50 units) of SuperScript II RT was added to each tube except to the ''no RT'' control tubes. The RT reaction was performed at 42^{o}C for 50 min and was then stopped by raising the temperature to 70^{o}C for 15 min. Then 1µl of RNase H was added and the mixture was incubated for 20 min at 37^{o}C. Subsequent PCR amplification was performed under standard conditions, and the RT-PCR products were visualized after 1.8% agarose gel electrophoresis.

2.8 Cloning and expression of tesB-like protein

2.8.1 Construction of the vector, growth of bacteria and preparation of cell extracts for thioesterase assay. The gene ABO_1111 encoding for tesB-like acyl- CoA thioesterase was amplified with the primers 1086F (5' – TTA CTG GCT TCG CAG GAA TGG– 3') and 1086R (5' – CTT GCT TAC CTA AAG TCC GCG– 3') and the PCR product was cloned into pCR2.1 Topo cloning vector (Invitrogen). The gene was excised from the resulted plasmid as *EcoRI* fragment and was ligated into *EcoRI* site of pCDFDuet-1 vector (Novagen) and transformed into E.coli DH5α competent cells (Invitrogen) and the transformants were selected on LB with streptomycin (50μg/ml). The clones were checked for the right orientation of the cloned gene and the positive plasmid was then transformed into RosettaBlue^{TM}(DE3)pLys competent cells (Novagen) and the transformants were selected on streptomycin (50μg/ml) and chloramphenicol (34μg/ml). *E. coli* was grown at 37 °C in Luria-Bertani (LB) medium containing 34 μg/ml chloramphenicol (for WT) or 34 μg/ml chloramphenicol plus 50 μg/ml streptomycin (for *tesB*-like gene expressing cells). When the OD_{600} reached 1.0, IPTG was added to a final concentration of 0.5 mM to induce expression for 2 hours, after which cells were harvested washed with 50 mM potassium phosphate buffer , pH 8.0 and stored at 4ºC, until use.

Approximately 0.5 g (wet weight) of *E. coli* cells expressing or not the *tesB*-like gene, were suspended in 1 ml of buffer A (50 mM potassium phosphate, pH 8.0, 10 mM EDTA, which was supplemented with 200 μg of phenylmethylsulfonyl fluoride and 5 μg DNase I grade II per ml) and disrupted by sonification for a total of 4 min (30 sec pulses, 1 min pauses) at 4°C in a W 250 sonifier (Branson Schallkraft GmbH, Germany). Soluble cell fractions were obtained as supernatants from 30 min of centrifugation at 15,000 *g* and 4°C. The resulting supernatants were monitored for thioesterase activity. The total protein concentration was determined by the Bradford method using BSA as standard.

2.8.2 Synthesis of (R,S)-3-Hydroxyacyl-CoA for thioesterase assay.

As a reference substances, (*R,S*)-3-hydroxyacyl-CoAs (from hexadecyl to decanoyl) were synthesized as described (Rehm et al., 1998) using 10 milliunits of acyl-CoA

synthetase (Sigma) in 100 ml of 50 mM Tris-HCl, pH 7.5, containing 2 mM ATP, 5 mM $MgCl_2$, 2 mM coenzyme A, and 2 mM (*R,S*)-3-hydroxyacid (hexanoate to decanoate). The reaction was stopped by the addition of 5 volumes of Dole's reagent (80% (v/v) isopropanol, 20% (v/v) *n*-heptane, 0.02 N H_2SO_4), and remaining free fatty acid was extracted with *n*-heptane. (*R,S*)-3-hydroxybutyryl-CoA was provided by Sigma.

2.8.3 Thioesterase assay.

The hydrolysis of acyl-CoAs and hydroxyacyl-CoAs was determined with a 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB)-based assay, as described elsewhere (Zhuang et al., 2000). Briefly, reactions were carried out in 50 mM potassium phosphate buffer, pH 8.0 with a 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB)-based assay in which 5-thio-2-nitrobenzoate, produced by the reaction of DTNB with the CoA liberated by hydrolysis of the acyl-CoA substrate, was monitored by its absorbance at 412 nm (molar extinction coefficient: 13600 M^{-1}). The 1 ml reaction mixture contained 4 µM acyl-CoAs (ranging from acetyl-CoA to decanoyl) or hydroxyacyl-CoA (ranging from acetyl to decanoyl), 1 mM DTNB and 100 µl of crude cell extract in a quartz cuvette of 1-cm light path length. One unit of enzymatic activity was defined as the amount of protein releasing 1 µmol of CoA per min.

2.9 2-DE of the cytoplasmic fraction

2.9.1 Preparation of the cytoplasmic protein fraction for 2-DE

Cultures were harvested by centrifugation at 4 °C at 8000 x *g* for 15 min, resuspended and washed twice in phosphate-buffered saline (Sambrook et al., 1989). The resulting pellet was stored at -20°C. For protein extraction, a cell pellet was allowed to thaw on ice, rehydration buffer (4 % CHAPS, 30mM DTT, 20 mM Tris Base, 7 M urea, 2 M thiourea, 0.2 % IPG buffer, one pellet of protease inhibitor cocktail (Complete™ Mini Boehringer, 20 ml^{-1}) was added and the suspension sonicated on ice with a 3.5 mm sonication probe (Labsonic U; Braun, Melsungen, Germany) six times (91 W, repeating cycle of 0.6 s), with 30 s intervals between each cycle. The microfuge tubes were centrifuged to remove the cell debris. For nucleic acids digestion, 1/1000 of Benzonase® Nuclease (Novagen) and $MgCl_2$ (2 mM final conc.) were added and the tubes were incubated at 4°C for 1 h. After that the extracts were transfered to centrifuge

tubes (Beckmann Polycarbonate) and centrifuged for 45 min at 4 °C (Beckmann Ultracentrifuge, 28 K, Rotor TLA 100.3). In order to remove salts, DNA and other contaminating substances the supernatants were extracted with cold phenol. To one volume of sample two volumes of phenol (equilibrated with TE-buffer, pH 7.4) and two volumes of water were added, vortexed vigorously, incubated on ice for 15 min and centrifuged. The aqueous phase was discarded without disturbing the white protein-containing lower phase. Two volumes of water were added and the procedure was repeated twice. For protein precipitation ice-cold aceton was added before centrifugation step. The resulting protein pellets were washed twice with ice-cold aceton, air-dried, resuspended in up to 500 µl rehydration buffer. The total protein concentration was determined by the Bradford method using BSA as standard (Bradford, 1976).

2.9.2 Isoelectric focusing and second dimension of the cytoplasmic fraction

Two-dimensional gel electrophoresis was carried out according to Görg et al.(1991). In the first dimension, each sample (500 µg in a total volume of 300 µl) was subjected to isoelectric focusing in IPG Ready Strips (17 cm) pH 3-6 or 4-7 (Bio-Rad, Munich, Germany). The gels were passively rehydrated for 2 h followed by an active rehydration step for 12 h at 50 V in rehydration buffer on a PROTEAN II Cell (Bio-Rad, Munich, Germany). Isoelectric focusing was performed at a maximum voltage at 5000 V for 150 Vh. For the second dimension the gels were equilibrated for two intervals of 15 min in equilibration solution (6 M urea, 30 % glycerol, 2 % SDS, 50 mM Tris base, pH 8.8). For the first step 2% DTT was added to the solution that was replaced by 2.5 % iodacetamide in the second round. The strips were then applied to 1.5 mm-thick gradient SDS-polyacrylamide (12 % -15 % w/v) gels. The second dimension was run approximately at 100 V in a IsoDalt system (Amersham Pharmacia Biotech, Uppsala, Sweden) overnight. The gels were stained with Collodial Coomassie brilliant blue day (CBB G-250) and analyzed. Proteins were identified by mass spectrometry using peptide fingerprints. Proteins were cut out of gels, destained and prepared for MALDI-TOF analysis, according to Wissing et al. (2000). The peptide mass fingerprints obtained were identified using an *A. borkumenis* SK2 protein data base that was build up in conjunction with a genome sequencing study.

2.10 2-DE of the membrane fraction

2.10.1 Preparation of the membrane fraction for 2-DE.

Cells of *Alcanivorax borkumensis* SK2 were grown on ONR7a medium at 30^{O}C. Cells from 400ml were harvested (4500 g x 20 min), and resuspended in 10 mL 100 mM Tris-HCl (pH 7.0) buffer, incubated at 37 °C in a shaking water bath for 3 h, and sonicated on ice for 20 min at 50% power and a duty cycle of 5 in a Branson Sonifier. The membrane and cytoplasmic fractions were separated by centrifugation at 30 000 g x 30 min at 4ºC. The membrane fraction was washed twice with buffer and stored at -70ºC, until use. The membrane fraction was prepared by the sarkosyl method of Filip *et al* (1973). Briefly, the membrane fraction was prepared as above, resuspended in an equal volume of buffer, 100 mM Tris-HCl (pH 7.0), containing 2% sodium-lauryl sarcosinate, 150 mM NaCl, and incubated at 37 °C for 1 h to facilitate inner membrane solubilization. To precipitate membrane proteins, 2 volumes of equilibrated phenol (AppliChem GmbH, Darmastadt, Germany) were added to 1 volume of sample, and the suspension was vigorously vortexed, incubated on ice for 10 min and centrifuged (16000 x *g*, 15 min, 4°C). The top aqueous phase was removed, 2 volumes of distilled water were added, and the mixture was vortexed, incubated on ice for 10 min and centrifuged (4000 x *g*, 15 min, 4°C). The aqueous phase was removed and the step repeated. Then 1 ml of ice-cold acetone was added. Tubes were inverted several times, incubated on ice for 10 min and centrifuged (16000 x *g*, 15 min, 4°C). The liquid phase was removed and the remaining pellet air-dried for 5-10 min. Pellets were suspended again in the solubilization solution and analysed by two-dimensional gel electrophoresis.

2.10.2 Isoelectric focusing and second dimension of the membrane fraction

2-DE was carried out as described previously (Heim *et al*., 2003). Briefly, approximately 200 µg of protein was applied to 24 cm pH 3-10NL IPG strips (ReadyStrip™, Bio-Rad, USA) and fractionated by isoelectric focusing on a Protean IEF Cell (Bio-Rad) at a maximum voltage of 10000 V for approximately 320 KVh according to the following program: 50 V, 100 Vh; 300 V, 800 Vh; 600 V, 2000 Vh; 2500 V, 5000 Vh; 7500 V, 30000 Vh; 10000 V until the end of run. The strips were then loaded on 1.5 mm thick 10-15% SDS-polyacrylamide gels and run overnight on a Hoefer DALT system (Amersham Biosciences). The gels were then fixed with 10% trichloroacetic acid and Coomassie-stained. Digitized images of Coomassie stained 2-D

gels were acquired by scanning. Protein spots were excised from preparative gels stained with Coomassie Brilliant Blue G250. *In situ* trypsin digestion (sequencing grade modified trypsin, Promega, Madison, WI, USA) and peptide extractions were performed as described previously (Wissing *et al*., 2000). Peptide samples were eluted from ZipTips® U-C18 (Millipore, Bedford, MA, USA) using 1.5 µl of saturated α-cyano-4-hydroxycinnamic acid (Sigma) and analysed by protein sequence using Quadrupole Time-of-Flight (Q-TOF) Mass Spectrometry.

2.11 Prediction of putative operon structures and putative promoters

Prediction of putative operons encompassing genes of interest (those found to be differentially expressed on alkanes) were done on the basis of close vicinity (less than 50 bp) of uniformly orientated genes encoding predicted functionally related proteins, and/or if a good putative promoter was found preceeding the first gene of the predicted operon. Putative sigma^{70}, σ^{54}, σ^{38}, and σ^{32}-dependent standard-type promoters of *Alcanivorax borkumensis* were identified by sequence homology to the published consensus sequences (Harley and Reynolds, 1987; Inouye 1987; Espinosa-Urgel et al., 1986; Cowing et al., 1985, correspondingly). We refrained from the search for promoters of other than the types listed above, as most of the putative operons of interest actually were preceeded by either of these promoter types, which in the context of predicting putative operon structure was considered sufficient.

2.12 Prediction of putative functions of novel proteins based on sequence homology

The putative functions for the proteins with low or no homology to the known proteins were predicted using some of many tools currently available on the web. Firstly, the amino acid sequences of the identified proteins were obtained from the *Alcanivorax borkumensis* SK2 genome and subjected to a BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) homology search. Many of the novel proteins showed a certain conserved domain at this stage. Secondly, the amino acid sequences were also aligned to the *A.borkumensis* genome itself (BLAST to self). Thirdly, and those with no functional domain were further analyzed by Pfam (Pfam, http://www.sanger.ac.uk/Pfam/). Finally, the protein sequences were also checked for the presence of transmembrane domains using the program Tmpred (Krogh et al., 2001). Information on the domain structure of predicted proteins was obtained by searching the protein sequence against the SMART detabase (Letunic et al., 2002).

3. RESULTS

3.1. Identification of genetic determinants of alkane metabolism in *Alcanivorax* strain SK2 by proteomics

Alcanivorax borkumensis strain SK2 is a cosmopolitan marine bacterium exhibiting a specialized metabolism adapted to the degradation of petroleum oil hydrocarbons. Therefore, it was of specific interest to identify the molecular basis of this organisms's ability to degrade alkanes. Initially, mini-Tn5 transposon mutagenesis was employed to screen for alkane degradation-deficient mutants and to map genes involved in the terminal oxidation of alkanes. However, this classical approach to apply random transposon mutagenesis to *A. borkumensis* SK2 and screen for mutants deficient for growth on alkanes, yielded down mutations only in genes responsible for steps further downstream in the degradation pathway of alkanes, but never in the initial oxidation steps, which are predicted to be catalysed by either one, or both, of two alkB monooxygenases (*alk*B1 and *alk*B2), found by *in silico* analysis of the genome of *Alcanivorax* SK2.

To identify the multiple routes involved in the primary degradation of alkanes, a complementary proteomic approach was employed. As a prerequisite of this study the appropriate range of pI for the two-dimensional gel electrophoresis had to be chosen. 2-DE profiles of *A. borkumensis* proteins on IPG strips from 3-10 have revealed that most of the cytoplasmic proteins lie in the area of pI 4-7 (data not shown), and we therefore focussed on this particular range of pI. Moreover, the average value of pI for all the putative proteins from the draft genome sequence of A. *borkumensis* was calculated to be around 6.0. Differentially expressed (up- or down-regulated or uniquely expressed under either condition) cytoplasmic proteins likely to be involved in alkane degradation by *A. borkumensis* SK2 were at first visualized by comparing two-dimensional gel electrophoresis images from cells in early stationary phase, growing with either pyruvate or hexadecane as sole carbon/energy source. The differentially expressed proteins were cored from the gels, digested *in situ*, and identified by MALDI MS analysis (for details see Table 1 and 2). Fig. 1 shows Coomassie-stained 2-D gels of the cytoplasmic fraction of *A. borkumensis* cells grown on either pyruvate (A) or hexadecane (B) as sole carbon and energy source. Expression of most of the cytoplasmic proteins was found not to be altered depending on the carbon source used. A total number of twenty five cytoplasmic proteins consistently showing differential

expression was detected (Table 1). Of these, seven spots (spots no 2, 6, 8, 9, 16, 21, and 22) appeared exclusively in hexadecane-grown cells: outer membrane protein OprF (spot 2C), phosphoenolpyruvate synthase PspA-1 (spot 6C), malic enzyme MaeB (spot 8C), isocitrate lyase AceA (spot 9C), 50S ribosomal protein RplY (spot 16C), 30S ribosomal protein RpsB (spot 21C) and a putative acyl-CoA dehydrogenase (spot 22C). In contrast, eleven spots were detected exclusively in pyruvate-grown cells: outer membrane protein (spot 1C), outer membrane receptor FecA (spot 4C), fimbrial assembly protein precursor PilQ (spot 5C), 2,4-Diaminobutyrate (DABA) aminotransferase EctB (spot 7C), NADH-dependent isocitrate dehydrogenase Icd (spot 11C), conserved hypothetical protein (spot 17C), LysM domain protein (spot 18C), phosphate ABC transporter PstS (spots 19 and 20), acyl-CoA dehydrogenase (spot 23C), and hypothetical protein (spot 24C). Identical MALDI spectra were obtained for the twin spots 17 and 51, 42 and 44, 6 and 83. Considerably increased expression in cells grown on alkane was detected for malate synthase GlcB (spot 10C; 4x), fatty acid oxidation complex alpha subunit (spot 12C; 61x) and cytochrome P450 (spot 25C; 1.9x), while hypothetical protein (spot 3C; 2.7x), acetyl-CoA carboxylase AccA (spot 13C; 2.2x), acetyl-CoA carboxylase AccC (spot 14C; 1.9x), and long-fatty-acid CoA ligase FadD (spot 15; 2.7x), were up-regulated in cells grown on pyruvate as carbon source.

For the membrane fraction we have chosen a wider range of pI (4-9). Taking into account the fact that most of the membrane proteins have rather extreme pI values, gel electrophoresis of membrane fractions from hexadecane-grown cells presented some problems with respect to getting clear 2-DE images which might be due to the very hydrophobic nature of the proteins expressed on hexadecane, thus affecting isoelectric focusing. To circumvent the resulting difficulties to differentiate protein expression based only on protein images of the two variants derived from pyruvate- or hexadecane-grown cells, we extracted all visible spots from both variants. Fig. 2 shows Coomassie-stained 2-D gels of membrane proteins of *A. borkumensis* grown on pyruvate (A) or hexadecane (B). Thirty eight spots were detected exclusively in hexadecane grown-cells: alkane 1-monooxygenase AlkB (spot 1M), outer membrane lipoprotein LolB (spot 2M), rubredoxin AlkG (spot 4M), hypothetical proteins (spots 3M, 5, 7-9M), ABC transporter (spots 6M and 83M), outer membrane protein OprG (spot 13M), regulator of the *alk*B1GHK operon (spot 14M), aldehyde dehydrogenase AlkH (spot 15), alcohol

dehydrogenase alkJ (spot 16M), medium-chain-fatty acid CoA ligase (spots 17M and 51M), succinate dehydrogenase SdhD (spot 22M), long-chain-fatty acid CoA ligase FadB (spot 23M), fatty acid oxidation complex FadB2 (spot 26M), (S)-2-hydroxy-fatty acid dehydrogenase RibD (spot 27M), permease protein (spot 29M), cardiolipin synthase (spot 30M), conserved hypothetical protein (spot 32M), 3-oxoacyl-[acyl-carrier-protein] synthase (spot 34M), fatty acid desaturase (spots 42M and 44M), poly-β-hydroxybutyrate polymerase PhaC (spot 45M), putative metabolite transport transmembrane protein (spot 49M), ABC transporters (spots 63M and 83M), putative membrane protein (spot 64M), multidrug/solvent RND membrane fusion protein (spot 67M), putative membrane-associated metalloprotease (spot 68M), putative lipoprotein (spot 73M), dihydroxy-acid dehydratase LlyD-1 (spot 75M), putative monooxygenase (spot 77M), alcohol dehydrogenase (spot 78M), nitrite extrusion protein NarK (spot 87M), sodium solute transporter family protein (spot 89M). In contrast, sixteen spots were found exclusively in pyruvate-grown cells: ectoin synthase EctC (spot 31M), outer membrane lipoprotein (spot 33M), inner membrane protein AmpE (spot 35M), putative membrane protein (spot 36M), putative outer membrane porin (spot 41M), outer membrane lipoprotein carrier protein LolA (spot 46M), ferric siderophore transport protein ExbD2 (spot 56M), hydrolase (spot 55M), membrane proteins (spots 61M and 65M), heavy metal RND efflux outer membrane protein CzcC (spot 62M), 2-oxoglutarate dehydrogenase LpdG (spot 74M), ABC transporter (spot 81M), IlvD2 (spot 82M), phosphate transporter (spot 85M), and oligopeptide ABC transporter (spot 88M). Identical Maldi spectra were obtained for the twin spots 19 and 20.

By combining the results from the two separate proteome maps of both membrane and cytoplasmic fractions of the cells a rather complete view on metabolic features of *A. borkumensis* during growth in alkanes was obtained. Of particular interest is the proteomic analysis of the membrane fraction, since it allowed the identification of many enzymes directly involved in the terminal oxidation of alkanes which are known to be membrane-bound (Whyte et al., 2002; Ratajczak et al., 1998; Smits et al., 2002). The identified differentially expressed proteins from both membrane and cytoplasmic fractions fall into a number of groups of functionally related proteins, often forming distinct gene clusters on the chromosome. These clusters were found to represent or include several putative operons, as defined by common orientation of genes, a maximum of 50bp between the genes succeeding each other (Goodchild et al., 2004)

and, in our case, by the presence of putative promoters. Analysis of the membrane proteins up-expressed on alkanes revealed that many of these appeared to be related to metabolic pathways directly involved or closely linked to the metabolism of alkanes, namely the terminal oxidation of alkanes, fatty acid oxidation, and polyhydroxyalkanoate production - the latter representing a major pathway storage route under conditions of excess carbon supply (Sabirova et al., submitted for publication). In contrast, cytoplasmic responses to growth on alkanes were found to mostly concern the activity of intracellular carbon fluxes (glyoxylate bypass, fatty acid synthesis, and fatty acid oxidation). Table 1 lists the identified differentially expressed proteins derived from both membrane and cytoplasmic protein fractions. For hypothetical, conserved hypothetical proteins and membrane proteins of unknown function listed in the Table 2, we have included some indications of predicted functions, as far as we could identify these by the use of various sequence analysis tools. A focused analysis of major complexes of metabolic pathways that, based on differentially expressed proteins listed in Table 1 and 2, found to be up-regulated upon growth on alkanes, is presented in section 4.1.

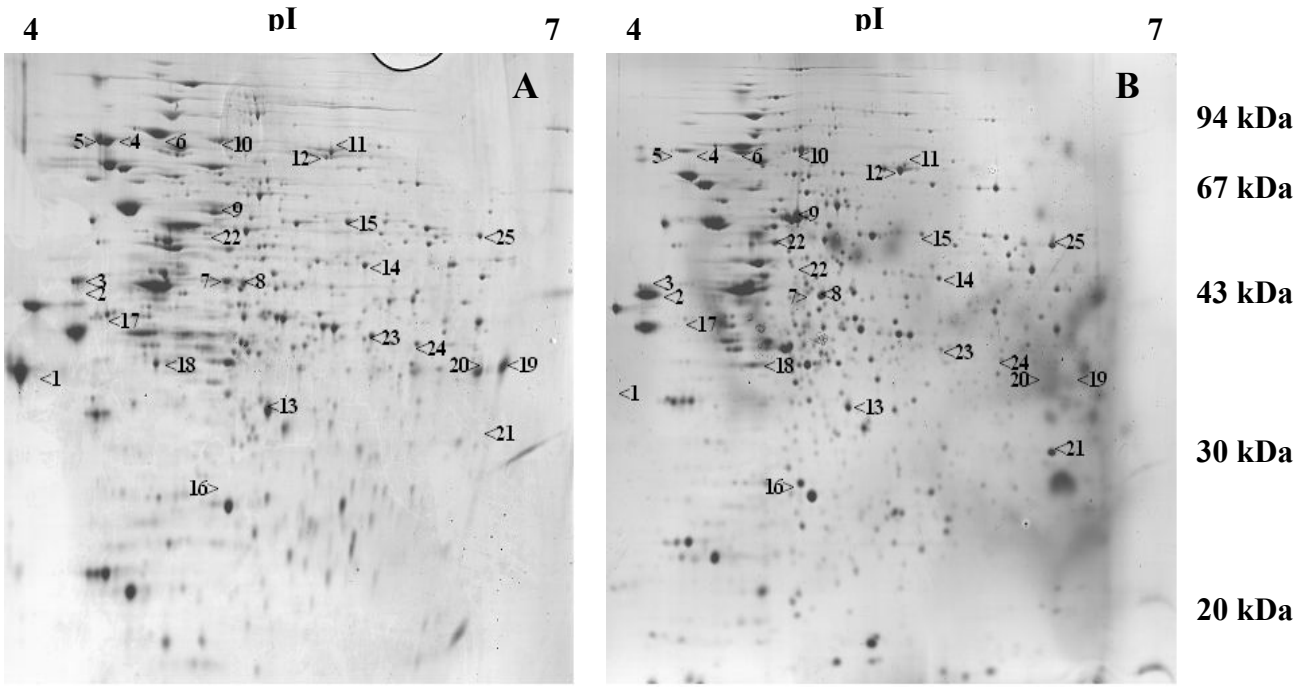


Fig.1. 2DE map of the cytoplasmic proteins of *Alcanivorax borkumensis* SK2 grown on pyruvate (A) or hexadecane (B). Isoelectric focusing was performed using IPG-strip of pH 4-7. Only proteins showing reproducible differentiall expression are numbered.

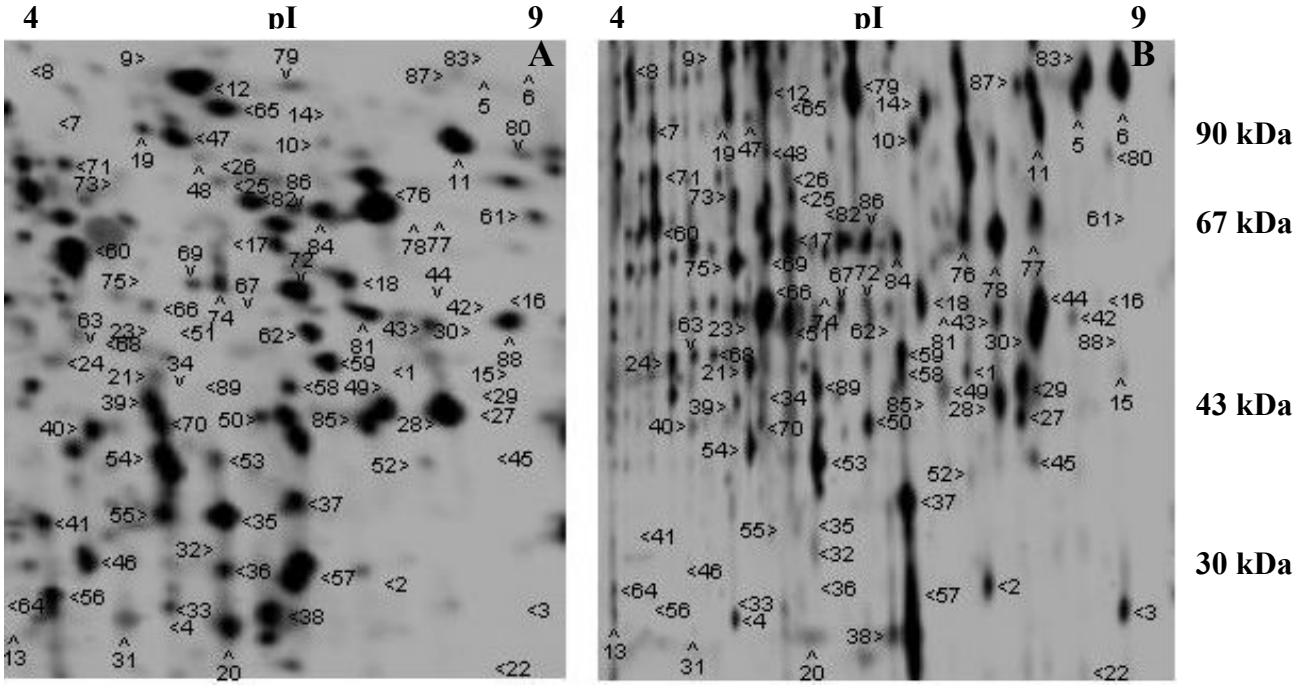


Fig.2. 2DE map of the membrane proteins of *Alcanivorax borkumensis* SK2 grown on pyruvate (A) or hexadecane (B). IEF was performed using an IPG strip of pH 4-9. All membrane proteins identified are numbered.

TABLE 1. Differentially expressed proteins derived from both membrane and cytoplasmic protein fractions of *Alcanivorax* SK2 grown on either hexadecane or pyruvate.

Spot no.	pI	MW	Gene function or functional category[1]	Gene number[1]	Differential abundance[2]	Putative operon[3]
Terminal oxidation of alkanes						
14M	6.4	98.1	Regulator of the alkB1GHJ operon (AlkS)	ABO_2706	H	1
1M	6.5	46.5	Alkane 1-monooxygenase (AlkB)	ABO_2707	H	1
4M	4.5	18.7	Rubredoxin (AlkG)	ABO_2708	H	1
15M	9.5	52.8	Aldehyde dehydrogenase (AlkH)	ABO_2709	H	1
16M	9.3	58.3	Alcohol dehydrogenase (AlkJ)	ABO_2710	H	1
77M	9.5	57.3	Monooxygenase (putative)	ABO_0190	H	2
78M	8.4	60.6	Alcohol dehydrogenase (AlkJ-2)	ABO_0202	H	3
25C	6.2	53.5	Cytochrome P450-1	ABO_0201	[4]	3
25C	6.2	53.5	Cytochrome P450-2	ABO_2288	[4]	-
Fatty acid oxidation						
23M	4.6	56.3	Long-chain-fatty-acid-CoA ligase (FadB)	ABO_0184	H	4
15C	5.4	62.1	Long-fatty-acid CoA ligase, putative (FadD)	ABO_0367	2.7 down	5
17M 51M	5.0	59.8	Medium-chain-fatty-acid CoA ligase (AlkK)	ABO_2748	H	-
22C	4.8	64.5	Acyl-CoA dehydrogenase (putative)	ABO_2102	H	-
23C	5.6	42.5	Acyl-CoA dehydrogenase	ABO_0988	7.2 up	6
26M	5.3	77.3	Fatty acid oxidation complex (FadB2)	ABO_1652	H	7
12C 25M	5.5	78.0	Fatty oxidation complex alpha subunit (FadB)	ABO_1566	61 up	8
Fatty acid and phospholipid biosynthesis						
13C	5.2	35.7	Acetyl-CoA carboxylase, carboxyl transferase, alpha subunit (AccA)	ABO_1159	2.2 down	-
14C	5.6	48.9	Acetyl-CoA carboxylase, biotin carboxylase (AccC)	ABO_2010	1.9 down	9
34M	5.0	42.7	3-Oxoacyl-[acyl-carrier-protein] synthase (FabB)	ABO_0834	H	10
53M	5.2	41.3	3-Oxoacyl-[acyl-carrier-protein] synthase (FabB)	ABO_1520	6.4 up	-
54M	4.9	42.1	3-oxoacyl-(acyl-carrier-protein) synthase (FabF)	ABO_1071	10.0 down	11
42M 44M	9.6	45.3	Fatty acid desaturase (putative)	ABO_2585	H	
52M	8.9	36.4	Sterol desaturase family protein	ABO_0114	2.3 down	-
30M	9.1	54.1	Cardiolipin synthase (Cls)	ABO_1816	H	-
Amino acid biosynthesis						
75M	4.9	58.5	Dihydroxy-acid dehydratase (IlvD-1)	ABO_0180	H	4
82M	5.2	70.9	Dihydroxy-acid dehydratase (IlvD-2)	ABO_2312	P	-
TCA, respiratory chain, glyoxylate bypass, and gluconeogenesis						
11C	5.5	82.6	Isocitrate dehydrogenase, NADH-dependent (Icd)	ABO_1281	2.0 down	12
74M	4.8	51.2	2-Oxoglutarate dehydrogenase (LpdG)	ABO_1494	P	13
55M	5.1	32.0	Hydrolase	ABO_1541	P	14
9C	4.9	59.1	Isocitrate lyase (AceA)	ABO_2741	36 up	-
10C	4.9	78.7	Malate synthase (GlcB)	ABO_1267	6.1 up	-
6C	4.7	86.9	Phosphoenolpyruvate synthase (PpsA-1)	ABO_1427	H	-
8C	5.0	45.8	Malic enzyme (MaeB)	ABO_2239	3.1 up	15
22M	8.8	13.2	Succinate dehydrogenase, hydrophobic membrane anchor protein (SdhD)	ABO_1499	H	16
76M	7.8	59.0	Oxidoreductase, GMC family	ABO_0187	5.6 up	4
Polyhydroxyalkanoate production						
45M	9.7	41.9	Poly-beta-hydroxybutyrate polymerase (PhaC)	ABO_1418	H	-
Osmoprotection						

31M	4.8	14.8	Ectoin synthase (EctC)	ABO_2152	P	17
7C	5.0	48.4	DABA aminotransferase (EctB)	ABO_2151	P	17
Cofactor synthesis						
27M	8.7	40.4	(S)-2-Hydroxy-fatty-acid dehydrogenase ribD,	ABO_2174	H	18
38M	6.2	27.3	Lipoil-(acyl-carrier protein)-protein-*n*-lipoyltrafsferase (LipB)	ABO_1963	26 down	19
Pilli formation						
5C	4.4	78.1	Fimbrial assembly protein preqursor (PilQ)	ABO_2233	46 down	20
Information processing and regulation						
21C	6.3	28.2	30S Ribosomal protein S2 (RpsB)	ABO_1143	H	21
16C	4.9	23.7	50S Ribosomal protein L25 (RplY)	ABO_0517	H	22
79M	6.5	91.6	Sensor histidine kinase	ABO_0442	14.0 up	-
Transport proteins						
6, 83M	9.0	87.1	ABC transporter, permease protein (putative)	ABO_1402	H	23
21M	4.8	47.1	ABC export system, membrane fusion protein	ABO_0248	7.2 up	24
63M	4.3	49.7	ABC export system, outer membrane protein	ABO_0250	H	24
81M	7.6	41.0	ABC transporter, ATP-binding protein, pernease (putative)	ABO_1847	P	25
84M	6.5	67.6	Oligopeptide ABC transporter, periplasmic peptide-binding protein	ABO_1219	5.2 down	26
88M	8.6	69.6	Oligopeptide ABC transporter, peripasmic peptide-binding protein	ABO_1220	P	26
19C 20C	9.2	37.3	Phosphate ABC transporter periplasmic binding protein (PstS)	ABO_2685	P	27
85M	8.7	44.3	Phosphate transporter (putative)	ABO_2305	P	-
89M	5.9	56.4	Sodium solute transporter family protein	ABO_1913	H	-
87M	9.7	95.9	Nitrite extrusion protein (NarK)	ABO_0547	H	28
49M	5.8	46.8	Metabolite transport transmembrane protein, putative	ABO_2038	H	-
24M	4.0	44.5	Long-chain fatty acid transporter, putative	ABO_0572	20 up	-
69M	5.0	62.5	Heavy metal RND efflux membrane fusion protein,CzcB family (CzcB2)	ABO_1357	3.1 up	29
62M	6.2	48.1	Heavy metal RND efflux outer membrane protein, CzcC family (CzcB1)	ABO_1358	P	29
59M	6.5	44.5	Heavy metal RND efflux membrane fusion protein, CZsB family (CzcB3)	ABO_1382	4.1 down	30
39M	4.6	40.7	Outer membrane polysaccharide export protein precursor (Wza)	ABO_0905	4.2 down	31
41M	3.9	36.1	Outer membrane porin (putative)	ABO_1621	P	-
4C 48M	4.4	80.2	FecA-like outer membrane receptor (FecA)	ABO_0721	43 down 2.5 up	-
56M	4.4	14.1	Ferric siderophore transport system, inner membrane protein E (ExbD2)	ABO_1968	P	32
46M	4.2	23.0	Outer membrane lipoprotein carrier protein (LolA)	ABO_1291	P	33
2M	8.8	21.8	Outer membrane lipoprotein (LolB)	ABO_0520	H	34
29M	8.6	45.6	Lipoprotein releasing system, permease protein, putative	ABO_1049	H	35
67M	5.6	47.8	multidrug/solvent RND membrane fusion protein (putative)	ABO_0965	H	36
68M	4.3	46.5	Membrane-associated zink metalloprotease (putative)	ABO_1150	H	37

TABLE 2. Differentially expressed proteins of uncertain or unknown function

Spot no.	pI	MW	Gene function or functional category[1]	Gene number[1]	Putative operon[3]	Differential abundance[2]	Putative function based on sequence analysis tools
20M	4.9	17.3	Putative membrane protein	ABO_0097	-	31 down	Membrane protein implicated in regulation of membrane protease activity.
18C	6.0	49.7	LysM domain protein	ABO_0132	-	4.1 down	Lysin domain, found in enzymes involved in bacterial cell wall degradation.
28M	7.1	42.2	Hypothetical protein	ABO_0154	-	2.8 down	No putative domains have been detected.
3M	9.4	23.7	Hypothetical protein	ABO_0160	-	H	No domains found
3C	4.2	48.7	Hypothetical protein	ABO_0193	38	2.7 down	similar to long-chain fatty acid transport protein of Marinobacter aquaeolei VT8.
33M	4.9	15.2	Outer membrane lipoprotein (OmlA)	ABO_0308	-	P	OmlA gene encodes a novel lipoprotein in *Ps. aeruginosa*. As in *Pseudomonas* the gene omlA is immediately upstream of divergently transcribed fur (ferric uptake regulator).
64M	3.7	19.9	Membrane protein (putative)	ABO_0443	-	H	ompA-like transmembrane domain is present in a number of different outer membrane proteins of several gram-negative bacteria.
73M	4.8	69.4	Lipoprotein (putative)	ABO_0586	39	H	Homologous to *lppC* gene. A part of a putative operon with gmhA gene (ABO_0584) encoding for phosphoheptose isomerase, required for lipooligosaccharide biosynthesis in other bacteria.
35M	6.2	31.9	Inner membrane protein (AmpE)	ABO_0621	40	P	The ampE gene encodes a transmembrane protein with uknown function. Upstream ampD gene (ABO_0620) encodes a cytosolic N-acetyl-anhydromuramyl-L-alanine amidase that participates in the intracellular recycling of peptidoglycan fragments.
65M	5.9	87.7	Membrane protein (putative)	ABO_0666	41	P	Predicted exporter of the RND superfamily
40M 2C	4.1	42.0	Outer membrane protein (OprF)	ABO_0822	-	55 down H	
61M	9.2	63.1	Membrane protein	ABO_0929	42	P	putative sodium-sulphate transporter. TM
66M	4.6	51.2	Membrane protein (putative)	ABO_0963	36	15.4 up	Outer membrane efflux protein. Forms trimeric channels that allow export of a variety of substrates in Gram negative bacteria.
7M	3.7	84.8	Conserved hypotetical protein	ABO_0997	-	H	No putative domains have been detected. TM
47M	4.9	84.6	Membrane protein (putative)	ABO_1242	-	12 up	His Kinase A (phosphoacceptor) domain.
36M	5.5	29.3	Membrane protein (putative)	ABO_1323	43	P	PEP:sugar phosphotransferase system, putatively involved in uptake of pyruvate in Alcanivorax. A part of a putative operon with the upstream gene (ABO_1322) encoding conserved hypothetical protein with pyruvate phosphate dikinase domain.
5M	8.5	101.8	Conserved hypotetical protein	ABO_1398	-	H	No putative domains have been detected. TM.

8M	3.5	108.8	Conserved hypotetical protein	ABO_1464	44	H	FimV, Tfp pilus assembly protein FimV. A part of an operon with the downstream truA gene in *Ps. aeruginosa*. The *fimV* gene is required for twitching motility while the *truA* gene is required for the type III secretory gene expression.
9M	4.8	105.3	Conserved hypotetical protein	ABO_1589	45	H	Family of proteins of unknown function.
32M	6.9	28.1	Conserved hypothetical protein	ABO_1588	45	H	Predicted spermidine synthase with an N-terminal membrane domain.
1C	3.9	36.1	Outer membrane protein	ABO_1621	-	P	Porins
24C	5.9	41.8	Hypothetical protein	ABO_1657	46	4.1 down	No putative domains have been detected.
12M	4.5	95.7	Conserved hypotetical protein	ABO_1823	47	4.1 down	No domains found. A part of a putative operon with the downstream genes. TM
13M	3.7	24.3	Outer membrane protein (OprG)	ABO_1922	-	H	A major outer membrane protein of *Ps. aeruginosa*; closest homology to *V.cholerae* ompW; is probably involved in low-affinity iron uptake (53).
57M	6.7	23.3	Membrane protein (putative)	ABO_1971	-	3.7 down	Predicted divalent heavy-metal cations transporter.
10M	7.0	86.1	Conserved hypothetical protein	ABO_2083	-	12.0 up	ANK, ankyrin repeats; ankyrin repeats mediate protein-protein interactions in very diverse families of proteins.
70M	5.2	39.8	Outer membrane phospholipase A precursor	ABO_2104	-	13 down	Outer membrane phospholipase A is an outer membrane-localized enzyme. It is implicated in the virulence of several pathogens.
17C	4.4	43.8	Conserved hypothetical protein	ABO_2153	-	4.6 down	No domains found.
72M	6.4	58.5	Membrane protein (putative)	ABO_2547	-	18 down	Uncharacterized iron-regulated membrane protein. TM
60M	4.8	65.1	Inner membrane protein	ABO_2753	48	4.3 down	This family of proteins is required for the insertion of integral membrane proteins into cellular membranes. Can be associated with respiratory chain complexes.

[1] - Gene numbers, gene function or functional category are presented according to the annotated genome (Schneiker et al., unpublished); [2] - H means that the protein is solely expressed on hexadecane; P means that the protein is solely expressed on pyruvate; ‘‘down’’ means that the protein is down-expressed on hexadecane; ‘‘up’’ means the protein is up-expressed on hexadecane; [3] – Putative operons demonstrated in this table are at least two consecutive genes encoding co-expressed functionally related proteins, closely associated on the chromosome and transcribed from their own putative promoter, identified by in silico analysis; [4] - For the expression pattern of the p450 cytochromes please refer to the text; [TM]- transmembrane domains (based on the TMHMM (Krogh et al., 2001).

3.2 Identification of genetic determinants of environmental adaptation of *Alcanivorax* strain SK2 by transposon mutagenesis

The strategy for random transposon mutagenesis in *Alcanivorax borkumensis* SK2 was based on the use of mini-Tn5 Str/Sp transposon element constructed by Lorenzo et al. (1990). A random mini-Tn5 transposon strategy was employed to screen for the genetic determinants of a number of phenotypes which are likely to play an important role in the adaptation of the marine oil-degrading bacterium *Alcanivorax borkumensis* SK2 to changing environmental conditions. The environmentally relevant phenotypes were screened for adaptation to high osmolarity, UV radiation, low temperatures, and the ability to form biofilms. A total of 48 mutants altered their ability to cope with any one of these specific environmental adaptations were isolated, and accordingly 48 genes of interest interrupted by mini-Tn5 insertions were sequenced by inverse PCR (Figure 3). The putative functions were assigned in adaptation to salt, UV, cold temperature and in the formation of biofilms, as listed in Table 3 and described.

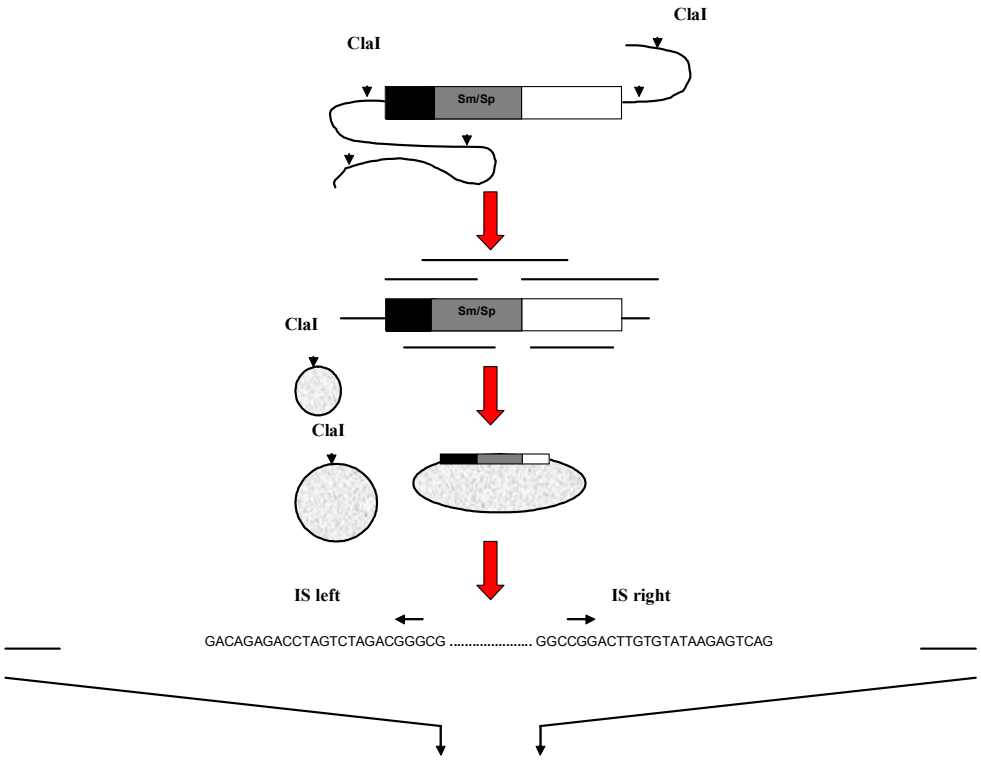


Figure 3. Schematic representation of inverse PCR reaction employed to amplify the regions flanking mini-Tn5 insertions. Shortly, the total DNA of the mutant was isolated and digested with *ClaI*, which does not cut mini-Tn5 element. The resulting DNA fragments were circularized with DNA ligase and the flanking regions of the inserted mini-Tn5 were amplified with two primers OTR End and 1TR End.

TABLE 3. The list of genes of *A.borkumensis* SK2 found by transposon mutagenesis to be environmentally relevant.

Gene name	Mutant name	Protein name	Phenotype
DNA repair			
ABO_0427	9H10	UvrA, excision nuclease ABC, A subunit	UV^S
ABO_0427	5H21	UvrA, excision nuclease ABC, A subunit	UV^S
ABO_0427	3O2	UvrA, excision nuclease ABC, A subunit	UV^S
ABO_0945	7E21	UvrB, excision nuclease ABC, B subunit	UV^S
ABO_0945	6A8	UvrB, excision nuclease ABC, B subunit	UV^S
ABO_1305	7I13	UvrC, excision nuclease ABC, C subunit	UV^S
ABO_1305	11L15	UvrC, excision nuclease ABC, C subunit	UV^S
ABO_1801	1D15	RecA protein	UV^S
ABO_0753	4L19	RuvB, Holliday junction DNA nuclease	UV^S
ABO_2538	6G17	Rep ATP-dependent DNA helicase	UV^S
ABO_1474	10D14	YfcB, site-specific DNA-methyltransferase	UV^S
Signal transduction and regulation			
ABO_2433	2A17	Conserved hypothetical signal transduction protein	UV^S
ABO_1690	10D13	PfeS, putative kinase sensor protein	UV^S
ABO_1987	10N15	DNA-binding response regulator, LuxR family	UV^S
ABO_2149	G5	Transcriptional regulator, MarR family	O^S
ABO_1986	12B12	Two-component sensor histidine kinase protein, putative	O^S
ABO_2269	3G22	GTP-binding regulator BipA/TypA	T^S
ABO_1835	5D12	Putative transcriptional regulator	T^S
ABO_1028	7H4	Mfd, transcription repair coupling factor	B^D
ABO_1986	6M17	Two-component sensor histidine kinase protein, putative	B^D
ABO_2691	2E3	Sensory box protein	B^D
ABO_2433	7O13	Conserved hypothetical signal transduction protein	B^D
Cell division			
ABO_2735	2A5	ParA, parA family ATPase	UV^S
Transport systems			
ABO_0291	10D18	ABC transporter, ATP-binding protein	UV^S
ABO_2146	4A15	TRAP dicarboxylate transporter, extracellular solute binding protein, DctP subunit	UV^S
ABO_0250	1N21	ABC export system, outer membrane protein	O^S
ABO_1673	D4	ABC efflux transporter, permease protein, putative	O^S
ABO_2623	6K16	MATE efflux family protein, putative	O^S
ABO_2631	3C7	mtdA, multidrug transporter	B^D
Biosynthesis of osmoprotectors			
ABO_2151	4P19	Diaminobutyrate-pyruvate aminotransferase, ectB	O^S
Changes in membrane composition			
ABO_0020	5J11	mmsA, methylmalonate semialdehyde dehydrogenase	T^S
mRNA degradation			
ABO_1768	5E16	Rnd, ribonuclease D, rnd	T^S
ABO_0333	6K2	Pnp, polyribonucleotide nucleotidyltransferase	O^S
Electron transport chains			
ABO_1089	11N15	Nitrate reductase, large subunit	UV^S
ABO_0037	3N2	Putative cytochrome b561 family	UV^S
ABO_2209	10E11	Iron-sulfur cluster-binding protein, putative	UV^S
ABO_2248	3G3	Ubiquinone biosynthesis protein, putative	B^D
Function in adaptation is unknown			
ABO_0908	1L23	WbpO, capsular polysaccharide biosynthesis protein	UV^S
ABO_0221	2A4	Type two secretion system protein	UV^S
ABO_1823	1B13	Conserved hypothetical protein	UV^S
ABO_2621	7F13	PykA, pyruvate kinase II	O^S
ABO_1141	5C3	GlnD, protein-pII uridylyltransferase	T^S
ABO_2346	5D8	Conserved hypothetical protein	T^S
ABO_1926	7F5	Conserved hypothetical protein	T^S
ABO_0666	5G22	Putative membrane protein	T^S
ABO_1957	2J23	Membrane-bound lytic murein transglycosylase B	B^D
ABO_2302	7O20	GlnE, glutamate-ammonia ligase adenylyltransferase	B^D
ABO_1111	3H4	Acyl-Co thioesterase II	B^D

UV^S – UV sensitive mutants; O^S – osmosensitive mutants; T^S – low temperature sensitive mutants; B^D – mutants deficient in biofilm formation.

3.2.1 Isolation and identification of UV sensitive mutants

The current paradigm for prokaryotic DNA replication, repair, and recombination is mainly based on studies of the DNA metabolism in *Escherichia coli*, an enteric organism with exposure to only limited changes in its natural environment. While the base composition of the *A.borkumensis* genome displaying a G:C content of the DNA of 53.4% (Yakimov et al., 1998), which is comparable to the one of *E.coli* with 50% G:C, differently from *E.coli*, *Alcanivorax* in its marine environmental habitat is constantly exposed to intense solar UV. Therefore, it is believed, that this organism will have developed specific mechanisms to protect itself and in particular its DNA metabolism against the damaging effects of UV. Because of its environmental lifestyle characterized by constant exposure to intense solar UV radiation, we believe that it was important to analyze DNA metabolism of this bacterium.

We employed transposon mutagenesis to identify genes important in adaptation of *Alcanivorax* to UV radiation. 4000 transposon mutants were screened for increased sensitivity to UV light by replica plating. The mutants were spread and irradiated with short-wavelength UV radiation (UVC) which gave 50% survival of the wild type. The bacteria received 5-second single UV treatment using an ultraviolet light C lamp (wavelength 254 nm). We have chosen this UV wavelength, because it is known to be the most effective type of radiation leading to damages of the DNA. Therefore, the aim of using this type of radiation was to hit genes directly involved in DNA metabolism and repair. Twenty three mutants, designated 9H10, 5H21, 3O2, 7E21, 6A8, 7I13, 11L15, 1D15, 4L19, 6G17, 10D14, 2A5, 10N15, 2A17, 10D13, 10D18, 4A15, 11N15, 3N2, 10E11, 1L23, 2A4, and 1B13 were found to fail to grow after UV exposure as described. The positions of the transposon insertions for all these mutants were determined by inverse PCR. Below these mutants are grouped according to their putative function in relation to UV stress.

A) UV sensitive mutants with defects in genes related to DNA metabolism. The mutants designated as 9H10, 5H21, 3O2, 7E21, 6A8, 7I13, 11L15, 1D15, 4L19, 6G17, 10D14, were deficient in genes ABO_0427, ABO_0945, ABO_1305, ABO_1801, ABO_0753, ABO_0249, ABO_1474 encoding proteins directly involved in DNA metabolism. The transposon insertions for seven of them (9H10, 5H21, 3O2, 7E21, 6A8, 7I13, 11L15) were found to be within different subunits of the UvrABC

excinuclease encoded by ABO_0427, ABO_0945, and ABO_1305, correspondingly. The sequence information from these strains reveals that these mutants have insertions at different locations of the three genes.

ABO_0427 encoding UvrA is a highly conserved protein showing highest homology to *Azotobacter vinelandii* (AvOP, 75% identity, 87% similarity) and *Pseudomonas putida* (KT2440, 75% identity, 88% similarity).

ABO_0945 encodes UvrB protein. Database searches show this protein to be highly conserved in bacteria, with highest homology found to *Marinobacter aquaeolei* (VT8, 74% identity, 86% similarity) and *Pseudomonas aeruginosa* homologues (PAO1, 73% identity, 87% similarity). Residues 10 to 426 encompass a so-called DEXDc domain, identifying ABO_0945 as a member of DEAD-like helicases superfamily. Members of this family include the DEAD and DEAH box helicases (SMART). Helicases are involved in unwinding nucleic acids. The DEAD box helicases are involved in various aspects of RNA metabolism, including nuclear transcription, pre mRNA splicing, ribosome biogenesis, nucleocytoplasmic transport, translation, RNA decay and organellar gene expression (SMART). Residues 460 to 546 encompass HELICc domain (helicase superfamily C-terminal domain). This domain again is characteristic for a wide variety of helicases and helicase related proteins.

ABO_1305 encodes UvrC protein. Database searches show this protein is well conserved in bacteria with highest homology to *Pseudomonas* species (*Ps.aeruginosa* PAO1, 51% identity, 70% similarity; *Ps.syringae pv. syringae* B728a, 51% identity, 69% similarity; *Ps. putida* KT2440, 50% identity, 69% similarity). SMART analysis has shown, that residues 16 to 97 contain a GIYc domain (GIY-YIG type nucleases), which interacts with UvrB protein during nucleotide excision repair. Residues 553 to 572 and 585 to 604 contain HhH1 and HhH2 (Helix-hairpin-helix DNA-binding motives) domains, respectively. According to SMART analysis, the HhH DNA-binding motives are present in prokaryotic and eukaryotic non-sequence-specific DNA binding proteins. ABO_1305 is likely to be a second gene in an operon with the preceeding ABO_1304 encoding a DNA-binding response regulator gasA, and it is followed by ABO_1306 encoding CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase, the enzyme of phospholipid biosynthesis.

One mutant strain, 1D15, showed a deficiency in ABO_1801 encoding recA protein, known to be involved in the SOS response. Database searches show it to be highly conserved showing highest similarity to the recA proteins of different *Pseudomonas* species (*Ps.stutzeri*, 87% identity, 95% similarity; *Ps.aeruginosa* PAO1, 86% identity, 94% similarity; *Ps.putida* KT2440, 86% identity, 94% similarity). Residues 56 to 227 contain a so-called AAA domain (AAA ATPases). This domain is part of proteins involved in energy-dependent protein degradation (Neuwald et al., 1999). BLAST analysis of the aminoacid sequence of the predicted ABO_1801 encoded protein to the *Alcanivorax* genome itself identified as closest, but only distantly related homologue a gene encoding the DNA repair protein radA (ABO_2185), with 27% identity and 34% similarity. ABO_1684 is arranged in a putative operon with the downstream located ABO_1800, encoding a regulatory protein recX. Therefore, the transposon mutation of the recA gene is likely to have a polar effect on the transcription of the downstream recX gene, rendering it possible that either of the two or both of these genes account for the mutant phenotype.

Mutant 4L19 was found to have a deficiency in ABO_0753 encoding a so-called Holliday junction DNA helicase ruvB, involved in DNA strand resolution and migration during the process of recombination. The protein is well conserved across bacterial species showing highest homology to ruvB of *Pseudomonas syringae* (*pv. phaseolicola* 1448A, 72% identity, 85% similarity) and *Acinetobacter* (ADP1, 77% identity, 88% similarity), and *Azotobacter vinelandii* (AvOP, 74% identity, 86% similarity). SMART analysis shows its residues to comprise AAA domain. The ABO_0753 is likely to be operon-arranged with the upstream genes ABO_0751 and ABO_0752, annoted as ruvC and ruvA.

Mutant strain 6G17 carried the mini-Tn5 mutation in ABO_2538 encoding ATP-dependent DNA helicase rep, which catalyses ATP dependent unwinding of double stranded DNA to single stranded DNA. Database searches show it to be highly conserved showing highest similarity to rep helicases of *Marinobacter aquaeolei* (VT8, 59% identity, 76% similarity), *Chromohalobacter salexigens* (DSM 3043, 56% identity, 74% similarity), and *Microbulbifer degradans* (57% identity, 77% similarity). BLAST analysis of the aminoacid sequence of the predicted ABO_2538 encoded protein to the

Alcanivorax genome itself identified as closest homologue a gene encoding uvrD DNA-dependent helicase (ABO_2690), with 36% identity and 54% similarity.

Mutant strain 10D14 carried the mini-Tn5 insertion in ABO_1474, encoding adenine-specific DNA-methyltransferase known to be involved in mismatch repair. Database searches revealed the protein to be homologous to adenine-specific methylase of *Legionella pneumophila* (subsp. pneumophila str. Philadelphia 1, 55% identity, 71% similarity), methylase of polypeptide chain release factors of *Vibrio sp.* (Ex25, 52% identity, 72% similarity). BLAST analysis of the predicted aminoacid sequence of the protein to the Alcanivorax genome itself found a protein encoding hemK methyltransferase with 36% identity and 47% similarity.

B) UV sensitive mutants with defects in electron transport chains

We have also identified three genes which encode different components of various electron transport chains, which might be associated with the resistance mechanisms towards oxidative stress generated by UV radiation. Mutants designated as 11N15, 3N2, and 10E11 were deficient in genes ABO_1089, ABO_0037, and ABO_2209, correspondingly.

ABO_1089 encodes a large subunit of nitrate reductase, which is the terminal component of the electron transport system and catalyzes the reduction of nitrate to nitrite in *E.coli* (Haddock and Jones 1977). Database search has found as most closest homologue nitrate reductase of *Methylococcus capsulatus* (str. Bath, 51% identity, 65% similarity), a probable nitrate reductase large subunit oxidoreductase protein of *Ralstonia solanacearum* (GMI1000, 52% identity, 64% similarity), an anaerobic typically selenocystein-containing dehydrogenase of *Microbulbifer degradans* (2-40, 49% identity, 64% similarity). BLAST analysis of the predicted amino acid sequence of the protein to the *Alcanivorax* genome itself identified as distantly related gene products a putative oxidoreductase encoded by ABO_0901 (25% identity, 40% similarity), nitrite reductase nirB encoded by ABO_1086 (24% identity, 39% similarity) and nitrite reductase nirB1 encoded by ABO_0855 (25% identity, 41% similarity). ABO_1089 is likely to be operon-arranged with the upstream genes ABO_1086 encoding nirB nitrite reductase, ABO_1087 encoding nirD nitrite reductase, ABO_1088 encoding nitrite reductase family protein, and the downstream ABO_1090 encoding a conserved

hypothetical protein. Transposon insertion in ABO_1089 might have a polar effect on the downstream gene ABO_1090, which encodes a conserved hypothetical protein with some similarity to an alkyl hydroxyperoxide reductase that shown to protect against peroxides and peroxynitrite in other bacteria (Guimaraes et al., 2005; Salunkhe et al., 2005).

ABO_0037 encodes a putative cytochrome of b561 family. This family includes apart from cytochrome b561 also related proteins like e.g. a nickel-dependent hydrogenases b-type cytochrome subunit. Database search has revealed it to be highly conserved with highest homology to a putative membrane protein of *Marinobacter aquaeolei* (VT8, 67% identity, 82% similarity), a hypothetical protein plu2094 of *Photorhardus luminescens* (subsp. laumondii TT01, 56% identity, 75% similarity), the nickel-dependent hydrogenase b-type cytochrome subunit of *Azotobacter vinelandii* (AvOP, 54% identity, 70% similarity), and the cytochrome b561 of *Microbulbifer degradans* (2-40, 51% identity, 68% similarity). BLAST analysis of the aminoacid sequence of the protein to *Alcanivorax* genome itself identified distantly related proteins ABO_0099 encoding cybB cytochrome b651 (30% identity, 49% similarity) and ABO_0058 encoding putative cytochrome b561 (26% identity, 48% similarity). The protein encoded by ABO_0037 contains four transmembrane domains at positions 13 to 35, 55 to 72, 92 to 114, and 147 to 169. ABO_0037 is immediately followed by ABO_0038 encoding conserved hypothetical protein and therefore the transposon insertion might have a polar effect on a downstream gene. The BLAST has revealed it to be a hypothetical periplasmic protein yceI, whose function is not yet known.

C) UV sensitive mutants with mutations in genes involved in signal transduction. We identified three mutants carrying the Tn5-induced knockouts in genes forming part of different signal transduction systems.

ABO_1987 encodes a regulatory protein of a two-component system, and it is associated with a sensor histidine kinase encoded by ABO_1986, located further upstream in the same operon. BLAST search has found it to be highly conserved among bacterial species and to contain a signal specific receptor domain, which is not only present in bacteria (CheY, OmpR, NtrC, and PhoB), but has also recently been found in eukaryotes (*Arabidopsis thaliana*). Database search has found as closest homologous genes a number of response regulators consisting of CheY-like receiver domain protein

of *Microbulbifer degradans* (2-40, 50% identity, 68% similarity), regulatory protein, LuxR:response regulator receiver of *Marinobacter aquaeolei* and of *Rhodopseudomonas palustris* (VT8, 51% identity, 68% similarity; BisB5, 51% identity, 68% similarity). SMART analysis has revealed two domains. Residues 8 to 121 contain the REC domain homologous to the CheY receiver domain. This domain contains a phosphorylation site that is phosphorylated by a histidine kinase. Residues 236-293 contain a HTH_LUXR (helix_turn_helix, Lux regulon) DNA-binding domain.

The second mutant showing a deficiency in a signal transduction system is mutant strain 2A17, where ABO_2433, encoding a conserved hypothetical signal transduction protein is interrupted. Database searches found this protein to exhibit highest homology to PAS:GGDEF proteins of *Ralstonia* species (*Ralstonia metallidurans* CH34, 48% identity, 66% similarity; *Ralstonia eutropha* JMP134, 47% identity, 65% similarity) and to a conserved hypothetical protein of unknown function of *Pseudomonas aeruginosa* (PAO1, 47% identity, 66% similarity). SMART analysis showed it to have a domain arrangement similar to that of the later described ABO_2691, with three PAS/PAC domains, one DUF1 (GGDEF), and one DUF2 (EAL) domain. PAS domains, are involved in many signalling proteins of archaea, bacteria and eucaryotes, where they are used as a signal sensor domain. PAC domains, often found to be associated with PAS domains and located C-terminal to PAS domains. Their putative role is to contribute to the 3-D PAS folding. PAS-PAC domains are present in a wide range of proteins shown to be involved in light, oxygen, and redox sensing, but also in some ion channel proteins (Ponting and Aravind, 1997; Zhulin et al., 1997). The DUF1 (GGDEF) domain is homologous to the adenylyl cyclase catalytic domain. This prediction correlates with the functional information available for two GGDEF-containing proteins, namely diguanylate cyclase and phosphodiesterase A of *Acetobacter xylinum*, which together regulate the turnover of cyclic diguanosine monophosphate. Several other proteins carrying this domain also carry aditional domains with functions related to the sensing of environmental signal. These include PleD, a response regulator protein involved in the ''swarmer-to-stalked'' cell transition in *Caulobacter crescentus,* and FixL, a heme-containing oxygen sensor protein. The DUF2 (EAL) domain is found in diverse bacterial signalling proteins. It is also called EAL after its conserved residues. The EAL domain is a good candidate for a diguanylate phosphodiesterase function, thus the couterpart of the DUF1-encoded function. The domain contains many conserved acidic

residues that could participate in metal binding and are lilely to form the phosphodiesterase active site. BLAST analysis of the aminoacid sequence of the protein to the *Alcanivorax* genome itself picked up ABO_2691, as the closest homologue, with 28% identity and 47% similarity.

A mutant designated as 10D13 bears the Tn5-mutation in ABO_1690 encoding a pfeS kinase sensor protein. Database search found the protein to be well conserved with the closest homologues being a two-component sensor protein of *Ps. aeruginosa* (PAO1, 49% identity, 63% similarity), and sensor histidine kinases of *Ps. fluorescens* and of *Ps. putida* (Pf-5, 45% identity, 61% similarity; KT2440, 45% identity, 60% similarity). Residues 7 to 29 constitute a transmembrane domain. Residues 181 to 232 form a so-called HAMP domain, known to interract with histidine kinases, adenylyl cyclases, methyl binding proteins and phosphatases. It is found in bacterial sensor and chemotaxis proteins as well as in eukaryotic histidine kinases. Residues 233 to 292 contain a HisKA (His Kinase A phosphoacceptor) domain, which is the site of dimerisation and phosphorylation by histidine kinases. Residues 339 to 449 encompass a HATPase_c (histidine kinase-like ATPases) domain, usually found in several ATP-binding proteins, like histidine kinase, DNA gyrase B, topoisomerases, heat shock protein HSP90, phytochrome-like ATPases and DNA mismatch repair proteins (SMART). BLAST of the aminoacid sequence of the protein to the *Alcanivorax* genome itself picked up as a distantly related gene ABO_0626, encoding a phosphotransferase (27% identity, 46% similarity) and ABO_0713 encoding a rstB sensor histidine kinase (28% identity, 50% similarity) as well as a number of other distantly related histidine kinases. ABO_1690 is immediately preceded by a DNA-binding response regulator pfeR (ABO_1691) and these two gene obviously forming a two-component regulatory system in turn are followed by exogenous ferric siderophor receptor and a TonB receptor gene, which is likely to be arranged in the same operon with the two regulatory genes.

D) UV sensitive mutants with deficiencies in various transport systems.

Defence against UV stress in Alcanivorax also appears to be associated with the function of several transporters. A mutant designated as 10D18 bears the transposon insertion in ABO_0291 encoding the ATP-binding protein of an ABC transporter, which is highly conserved in other bacterial species, however being named differently in these organisms, e.g. ABC transporter of *Azotobacter vinelandii* (AvOP, 82%of

identity, 92% similarity), ABC transporter-like protein of *Pseudomonas fluorescens* (PfO-1, 82% identity, 91% similarity), or ATP-binding protein of ABC transporter of *Ps. putida* (KT2440, 81% identity, 91% similarity). Residues 56 to 275 and 374 to 540 contain two AAA domains. BLAST of the aminoacid sequence of the protein to the *Alcanivorax* genome itself picked up a number of other distantly related ATP-binding proteins of ABC transporters (data not shown).

Another mutant designated 4A15 bears the transposon insertion in ABO_2134 encoding the extracellular solute binding protein of the TRAP dicarboxylate transporter. This family of transporters forms a distinct class of receptor-dependent secondary transporters where the driving force for intracellular solute accumulation is an electrochemical ion gradient and not ATP hydrolysis (Kelly and Thomas, 2001). The protein is highly homologous to the corresponding protein of *Chromohalobacter salexigens* (DSM343, 68% identity, 82% similarity) as well as to the C4-dicarboxylate transport systems of *Alkalilimnicola ehrlichei* (MLHE-1, 46% identity, 66% similarity) and of *Marinobacter aquaeolei* (VT8, 38% identity, 60% similarity). BLAST of the aminoacid sequence of the protein to the *Alcanivorax* genome itself picked up a second homologous gene, ABO_2689, also encoding TRAP-type transport system. Interestingly, ABO_2689 is located directly downstream from ABO_2690 encoding uvrD and may be connected with it in function.

Finally, we have found one mutant designated as 1L23, which harbors transposon insertion in ABO_0908 encoding capsular polysaccharide biosynthesis protein WbpO. Database search revealed this protein to be highly conserved with the closest homologue being the polysaccharide biosynthesis protein of different *Bordetella* species, with highest homology to *Bordetella bronchiseptica* (RB50, 76% identity, 88% simiarity). BLAST of the aminoacid sequence of the protein to the *Alcanivorax* genome itself picked up a distantly related gene ABO_0384 encoding algD GDP-mannose 6-dehydrogenase (24% identity, 42% similarity). The gene ABO_0908 is located in the vicinity of other genes involved in ramnose biosynthesis, and this proximity may be of functional relevance.

3.2.2 Isolation and identification of mutants sensitive to low temperature

As a prerequisite for the screening fot cold sensitive mutants, the minimum temperature still permitting growth of the bacterium was determined to be 10 degrees C. To identify genes that are essential for growth at a low temperature, we set out to isolate cold-sensitive mutants of the bacterium that are deficient in growth at 10°C, but that grow normally at 30°C, the temperature optimum for this bacterium. Thus screening transposon mutants, generated by random insertional mutagenesis with mini-Tn*5*-Str/Sp, 40 putative cold-sensitive mutants were initially obtained. Upon more thourough subsequent analysis, only some of the original 40 mutants exhibited a truly severe cold-sensitive phenotype, and for 7 of these mutants the sites of insertion of the mini-Tn5 transposon insertion sites were identified by inverse PCR. The mutants obtained and the respectively interrupted genes are listed in the Table 3.

A) Cold sensitive mutants deficient in regulatory genes.

Two of the cold-sensitive mutants were found to be deficient in regulatory genes.

A mutant called 3G22 was found to be interrupted in ABO_2269, annotated as GTP-binding regulator TypA/BipA. Database searches found this protein to be highly conserved across bacterial species, with highest homology to the GTP-binding elongation factor family protein of *Acinetobacter* (ADP1, 74% identity, 86% similarity), to the GTP-binding protein TypA of *Psychrobacter cryohalolentis* (K5, 74% identity, 85% similarity) and of *Marinobacter aquaeolei* (VT8, 73% identity, 85% similarity), as well as to the GTP-binding protein TypA/ BipA of *Haemophilus influenzae* (86-028NP, 74% identity, 86% similarity), predicted to function as a membrane GTPase involved in stress response by *Haemophilus influenzae* (R2846, 74% identity, 86% similarity). Elongation factors belong to a family of proteins that promote GTP-dependent binding of aminoacyl-tRNAs to the A site of ribosomes during protein biosynthesis, and that catalyse the translocation of the synthesised protein chain from the A to the P site (pFam database). BLAST of the aminoacid sequence of the protein to the *Alcanivorax* genome itself picked up ABO_1634 encoding GTP-binding protein LepA (26% identity, 46% similarity), that is probably not functionally related to the ABO_2269 gene product, apart from the GTP-binding function.

Another regulatory cold-sensitive mutant designated 5D12 was deficient in ABO_1835 which encodes a putative transcriptional regulator. Database searches revealed this protein to be highly conserved in other bacterial species, with the highest homology to

transcriptional regulator of the MerR family of different *Pseudomonas* strains (Ps. aeruginosa UCBPP-PA14, 87% identity, 91% similarity; *Ps. fluorescens* PfO-1, 82% identity, 89% similarity; *Ps. syringae pv. phaseolicola* 1448A, 80% identity, 88% similarity), and of *Marinobacter aquaeolei* (VT8, 77% identity, 87% similarity), as well as to another predicted transcriptional regulator of *Microbulbifer degradans* (2-40, 78% identity, 86% similarity). SMART analysis showed that residues 19 to 89 encompass an HTH_MERR domain. (helix-turn-helix, mercury resistance). Based on sequence similarity, all of these proteins are predicted to function as transcription regulators that mediate responses to stress in eubacteria. They belong to the MERR superfamily of transcription regulators that promote transcription of various stress regulons by reconfiguring the operator sequence located between the -35 and -10 promoter elements (BLAST). BLAST of the aminoacid sequence of the protein to the *Alcanivorax* genome itself picked up two more transcriptional regulators (ABO_0244 and ABO_0765) also belonging to the MerR family of regulators.

B) Mutants deficient in membrane functions and transporters

There are several mutants that seem to have different membrane or transport functions (branched-chain fatty acid composition, permeases, transporters) affected. A mutant designated 5J11 bears the transposon mutation in ABO_0020, encoding methylmalonate semialdehyde dehydrogenase mmsA, which forms in turn a part of an operon encoding enzymes involved in the degradation of branched-chain aminoacids as a precursor for anabolic pathways of membrane components. Methylmalonate semialdehyde dehydrogenase of *Alcanivorax* is highly homologous to the respective enzymes of different marine bacteria like *Idiomarina loihiensis* (L2TR, 74% identity, 87% similarity), *Pseudoalteromonas atlantica* (T6c, 74% identity, 86% similarity), and *Shewanella oneidensis* (MR-1, 75% identity, 85% similarity). BLAST of the aminoacid sequence of the protein to the *Alcanivorax* genome itself picked up ABO_0962 encoding an aldehyde dehydrogenase family protein (33% identity, 50% similarity). The mutation presumably causes a polar effect on the transcription of the downstream genes. The downstream genes of the putative operon are ABO_0021 encoding acyl-CoA dehydrogenase acdA and ABO_0022 encoding 3-hydroxyisobutyrae dehydrogenase mmsB. The operon regarded to be involved in the generation of membrane components, is preceeded by the divergently orientated ABO_0019 encoding transcriptional regulator mmsR, which belongs to a family of transcriptional regulators

presenting arabinose-binding and dimerisation domain characteristic of AraC regulatory protein (BLAST), shown to be a positive regulator of the mmsAB operon in *Pseudomonas aeruginosa* PAO1 (Steele et al., 1992).

Mutant strain 5D8 bears the transposon mutation in ABO_2346, encoding a conserved hypothetical protein, which according to a BLAST search is predicted to be a putative permease. Database search found conserved hypothetical protein of *Pseudononas aeruginosa* (PAO1, 54% identity, 70% similarity) as the closest homologous protein. The protein contains 8 transmembrane domains at positions 7-38, 53 to 70, 83 to 105, 109 to 128, 141 to 163, 185 to 205, 217 to 236, and 246 to 268. The gene is immediately followed by ABO_2345 encoding prolipoprotein diacylglyceryl transferase lgt. Lgt is known to be involved in processing of lipoproteins, which are a subgroup of secreted bacterial proteins characterized by a lipidated N-terminus (Sander et al., 2004).

Mutant 7F5 carries ist mutation in yet another conserved hypothetical protein (ABO_1926), which Blast analysis hints at to be a putative protein-S-isoprenylcysteine methyltransferase, involved in post-translational modification (modification of C-terminal cysteines) of a distinct group of proteins and polypeptides, including the a-factor mating pheromone and RAS proteins of *Saccharomyces cerevisiae* (Anderson et al., 2005). Residues 13 to 30, 45 to 67, and 88 to 110 contain transmembrane domains. BLAST of the aminoacid sequence of the protein to the *Alcanivorax* genome itself picked up distantly related branched-chain amino acid ABC transporter (ABO_2510, 25% identity, 50% similarity).

ABO_0666 encoding putative membrane protein was found to be interrupted by transposon mutation in mutant 5G22. Blast search showed it to be highly homologous to exporter proteins of the RND family of *Marinobacter aqualeolei* (VT8, 99% identity, 99% similarity), of *Rubrivivax gelatinosus* (PM1, 47% identity, 67% similarity) and of *Azoarcus sp.* (EbN1, 48% identity, 67% similarity). There are 11 transmembrane domains. BLAST of the aminoacid sequence of the protein to the *Alcanivorax* genome itself picked up two other distantly related putative membrane proteins (ABO_1002 and ABO_1242). The gene is likely to be the last gene of an operon comprising the following upstream genes: two conserved hypothetical proteins (ABO_0674 and ABO_0673), three different oxidoreductases (ABO_0672, ABO_0671, and

ABO_0670), a hypothetical protein (ABO_0669), a hydrolase (ABO_0668), and a putative BNR domain-containing protein (ABO_0667). The putative operon is preceded by a divergently transcribed transcriptional regulator tetR (ABO_0675). Oxidoreductase (ABO_0670) contains a 2Fe-2S iron-sulfur cluster. Iron-sulfur proteins play an important role in electron transfer processes and in various other enzymatic reactions. The family includes plant and algal ferredoxins, which act as electron carriers in photosynthesis and ferredoxins, which participate in redox chains. The oxidoreductase encoded by ABO_0671 resembles phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases. ABO_0667 encodes a putative BNR-containing domain protein, which according to the Blast analysis is related to a plant photosystem II stability/assembly factor. ABO_0668 encodes a hydrolase acting on carbon-carbon bonds in ketonic substances.

C) Cold sensitive mutants deficient in various cellular functions.

Here those cold-sensitive mutants are described which do not apparently belong to those groups discussed above, found to be defective in either regulatory (group A) or membrane function (group B).

A mutant designated 5E16 bears the Tn5 mutation in ABO_1768 encoding ribonuclease D, an enzyme known to be responsible for RNA degradation and maturation. The protein is homologous to ribonuclease D of different *Pseudomonas* species. Residues 3 to 168 contain a so-called 35EXOc (3'-5' exonuclease) domain. Such a domain is also responsible for the 3'-5' exonuclease proofreading activity of *Escherichia coli* DNA polymerase I (polI) and other enzymes, it catalyses the hydrolysis of unpaired or mismatched nucleotides (according to the SMART analysis). Residues 207 to 286 contain an HDRC (helicase and RNase D C-terminal) domain, which has a putative role in nucleic acid binding. ABO_1768 is presumably the first gene of a putative operon comprising 5 genes, and therefore the mutation probably causes a polar effect on the expression of the downstream genes encoding three conserved hypothetical proteins belonging to a single family of however unknown function (ABO_1767, ABO_1766, and ABO_1765), and another protein encoded by ABO_1764, which belongs to the superfamily of so-called HTH proteins (histidine triad hydrolases) that act on the alpha-phosphate of ribonucleotides.

A mutant designated ABO_1141 bears the mini-Tn5 induced mutation in protein-pII uridylyltransferase encoded by the glnD gene. This enzyme belongs to nitrogen regulation (ntr) system, which regulates ammonia assimilation through modulating glutamate synthetase activity. The protein is homologous to the same protein of *Azotobacter vinelandii* (AvOP, 55% identity, 72% similarty) and of *Pseudomonas aeruginosa* (PAO1, 55% identity, 72% similarity). Residues 454 to 601 contain a HDc (characteristic for metal dependent phosphohydrolases) domain. These phosphohydrolase enzymes appear to be involved in the nucleic acid metabolism, signal transduction and possibly many other functions in bacteria, archaea and eukaryotes. *In silico* analysis of the genes located downstream from the mutation site revealed this gene to be a part of a gene cluster presumably involved in biosynthesis of diaminopimelate. The structure of the gene cluster is similar to the one of *E.coli*, described by Kim et al. (1998) and consists of ABO_2117 encoding methionine aminopeptidase, ABO_1141 encoding protein-pII uridylyltransferase glnD, ABO_1140 encoding putative N-succinyldiaminopimelate aminotransferase, ABO_1139 encoding arsC related protein, ABO_1138 encoding tetrahydrodipicolinate N-succinyltransferase, and ABO_1137 encoding succinyl-diaminopimelate desuccinylase.

3.2.3 Isolation and identification of salt sensitive mutants

As a prerequisite for the screening for osmosensitive mutants, the maximum concentration of NaCl in the medium still permitting growth of the bacterium was determined to be 6%. To identify genes that are essential for growth at osmotic up-shift, we set out to isolate osmo-sensitive mutants of the bacterium that are deficient in growth at 6% NaCl in ONR7a medium, but that grow normally in standard ONR7a, where NaCl concentration was calculated to be around 3%. Thus screening transposon mutants, generated by random insertional mutagenesis with mini-Tn*5*-Str/Sp, putative osmosensitive mutants were obtained.

A) Osmosensitive mutants with mutations in genes involved in signal transduction and gene regulation

Transposon mutagenesis has revealed two genes involved in regulation and signal transduction of osmoadapatation in *Alcanivorax*. Mutant G5 carried a transposon mutation in ABO_2149 encoding a transcriptional regulator of the MarR family.

Database search found it to be a conserved transcriptional regulator of the Mar family, also found in *Marinobacter aquaeolei* (VT8, 61% identity, 82% similarity) and in *Vibrio* species (*V. parahaemolyticus* RIMD 2210633, 53% identity, 81% similarity; *V. vulnificus*, 53% identity, 79% similarity). The SMART program revealed that it has a so-called MARR domain (residues 40 to 140). The marR-type HTH domain is a DNA-binding, winged helix-turn-helix domain present in transcription regulators of the marR/slyA family, involved in the development of antibiotic resistance. This family of transcription regulators is named after Escherichia coli marR, a repressor of genes which activate multiple antibiotic resistance and oxidative stress regulons (according to SMART). Alignement of the amino acid sequence of the protein against the Alcanivorax genome identified another distantly related protein encoding a transcriptional regulator of the MarR family, with 24% identity and 45% similarity (ABO_0014). ABO_2149 proceeds a cluster of downstream genes putatively involved in ectoin synthesis in *Alcanivorax*, including ectA (ABO_2150), ectB (ABO_2151), and ectC (ABO_2152). Therefore, ABO_2149 is likely to constitute one operon together with these downstream genes, and the mini-Tn5 insertion in ABO_2149 may have a polar effect on their expression. Moreover, there is another divergently transcribed cluster of genes comprising dctQ (ABO_2148), dctM (ABO_2147), encoding a TRAP-type C4-dicarboxylate transport system, whose promoter region may also be affected by the transposon mutation of the ABO_2149.

ABO_1986, encoding a sensor histidine kinase belonging to a two-component regulatory system was found to be interrupted in mutant strain 12B12. This same two-component system is also involved in UV adaptation in *Alcanivorax* SK2, as evidenced by a mutation described in the section 3.2.4.

B) Osmosensitive mutants with mutations in different transport systems.

Osmoadaptation seems to involve the functioning of different transport systems, three of which have been found by transposon mutagenesis to affect osmoadaptation in *Alcanivorax.* ABO_0250 encoding outer membrane protein of ABC export system was found to be interrupted in a mutant strain 1N21. Database search found as closest homologous protein a putative outer-membrane immunogenic protein precursor of *Bradyrhizobium japonicum* (USDA 110, 36% identity, 45% similarity), an RND efflux system outer membrane lipoprotein, NotT of *Bradyrhizobium sp*. (BTAi1, 35% identity,

55% similarity), and another such putative outer membrane efflux protein of *Rhodopseudomonas palustris* (CGA009, 35% identity, 56% similarity). Amino acid sequence of the protein aligned to the *Alcanivorax* genome itself revealed the presence of another homologous gene, ABO_0963, encoding a putative membrane protein. The ABO_0250 is likely to be operon-arranged with upstream located putative genes ABO_0248 encoding a membrane fusion protein of an ABC export system, and ABO_0249, encoding a putative ATP-binding protein/permease of ABC transporter.

Another transport system, i.e. putative ABC efflux transporter / permease protein encoded by ABO_1630 seems also to be inolved in osmoadaptation. The corresponding transposon mutant is denominated D4. Database search revealed as closest homologues an ABC-type antimicrobial peptide transport system/permease of *Pseudomonas aeruginosa* (UCBPP-PA14, 45% identity, 63% similarity) and of *Vibrio cholerae* (0395, 44% identity, 65% similarity). The protein encoded by the putative ABO_1673 contains four transmembrane domains. The ABO_1673 is likely to be operon-arranged with an upstream gene (ABO_1674), encoding an ATP-binding protein of ABC transporter, and with a downstream gene (ABO_1672), encoding a conserved hypothetical protein. Therefore the mini-Tn5 element may cause a polar effect on the expression of the downstream located ABO_1672.

One more transport system, displaying impaired osmoadaptation in a respective transposon mutant strain 6K16, was found to be in ABO_2623, encoding a putative MATE efflux family protein. Closest homologous proteins were found to be the multiantimicrobial extrusion proteins MatE of *Chromohalobacter salexigens* (DSM 3043, 62% identity, 77% similarity) and of *Marinobacter aquaeolei* (VT8, 46% identity, 64% similarity). SMART analysis revealed that the ABO_2623-encoded putative protein contains 11 transmembrane domains. Alignment of the amino acid sequence of the protein to the *Alcanivorax* genome itself revealed the presence of another homologous gene, ABO_0158, also encoding a putative protein belonging to a putative MATE efflux family protein.

C) Osmosensitive mutants with deficiencies in other cellular functions.

Osmoadaptation in *Alcanivorax*, like that of other bacteria is likely to require the synthesis of the osmoprotector ectoin, as evidenced by one of the mutants, 4P19, found to be deficient in ABO_2151, encoding an ectB diaminobutyrate-pyruvate aminotransferase, known to catalyse the second step in the biosynthesis pathway of ectoin (Peters et al., 1990; Tao et al., 1992). Database search revealed as its closest homologous gene the L-2,4-diaminobutyric acid acetyl transferase of *Methylomicrobium sp.* (AM01, 68% identity, 84% similarity), the L-2,4-diaminobutyric acid transaminase of *Methylobacter alcaliphilus* (64% identity, 80% similarity), and the 4-aminobutyrate aminotransferase as well as a related aminotransferase of *Microbulbifer degradans* (2-40, 61% identity, 76% similarity). Allignment of the predicted amino acid sequence of ABO_2151 against *Alcanivorax* genome itself revealed as a homologous gene a putative aminotransferase encoded by ABO_0509 (29% identity, 50% similarity). ABO_2151 is likely to be operon-arranged with the upstream located gene ectA (ABO_2150) and a downstream located gene ectC (ABO_2152).

Another osmosensitive Tn5 mutant strain 7E13 is mapped in ABO_2621, which encodes pyruvate kinase II pykA. The database search revealed as closest homologous genes the pyruvate kinases of *Chromohalobacter salexigens* (DSM 3043, 56% identity, 74% similarity), of *Marinobacter aquaeolei* (VT8, 58% identity, 73% similarity), and of *Azotobacter vinelandii* (AvOP, 59% identity, 72% similarity).

ABO_0333, bearing a Tn5 mutation in the osmosensitive mutant strain 6K2, encodes a polyribonucleotide nucleotidyltransferase pnp. Polynucleotide phosphorylase (PNPase) is a 3'-5'-exoribonuclease that catalyzes the phosphorolytic degradation of RNAs in *Escherichia coli* and other bacteria (Deutscher, M. P., and N. B. Reuven. 1991; Donovan and Kushner. 1986). The database search revealed as its closest homologous genes the RNA binding S1:KH proteins of *Marinobacter aquaeolei* (VT8, 69% identity, 80% similarity), and of *Ps. putida* (F1, 69% identity, 81% similarity), as well as a polyribonucleotide nucleotidyltransferase of *Pseudomonas putida* (KT2440, 69% identity, 81% similarity). SMART analysis found two distinct domains in the predicted putative operon: residues 554 to 619 comprise a KH (K homology RNA-binding)

domain, present in diverse nucleic acid-binding proteins and suspected to bind RNA (according to SMART), and residues 622 to 692 constitute a so-called S1 (ribosomal protein S1-like RNA-binding) domain. The S1 domain was originally identified in the ribosomal protein S1, but it is also found in a large number of other RNA-associated proteins. The structure of the S1 domain is very similar to that of cold shock proteins, suggesting that these two proteins may both be derived from an ancient nucleic acid-binding protein (according to SMART).

3.2.4 Isolation and identification of biofilm deficient mutants

Bacterial biofilm formation has recently been extensively been studied mostly on pathogenic bacteria, where it is supposed to play a major role in virulence and pathogenecity. Microscopic observation of *Alcanivorax* SK2 indicated that this organism forms biofilms preferably on ogranic substrates, which corresponds well to the needs of this microorganism. Thus, the biofilms were formed by *Alcanivorax* cells on Permanox slides, when studied in more detail using EM microscopy. The EM images show that biofilm morphology and its development was greatly dependent on the carbon source used, and that the stage of the biofilm changed over time. Thus after 4 days of growth, pyruvate-grown bacteria were still in the monolayer state, while alkane-grown bacteria were already in the biofilm stage. In case of pyruvate the *Alcanivorax* SK2 cells attached to the carrier surface changing the shape of the cells, which turned flatter, and cells tended to contact each other laterally. In contrast, cells grown on alkane are shorter and rounder, they connect to other either via their poles and are often arranged in chains, and they produce considerable amount of extracellular polymeric substances (EPS), which appears to support the 3-dimensional structure of the biofilm. After 10 days of growth the alkane-grown cells had developed a biofilm, that exhibits a pronounced three-dimensional architecture consisting of double-cell chains and supported by extracellular matrix (Figure 4).

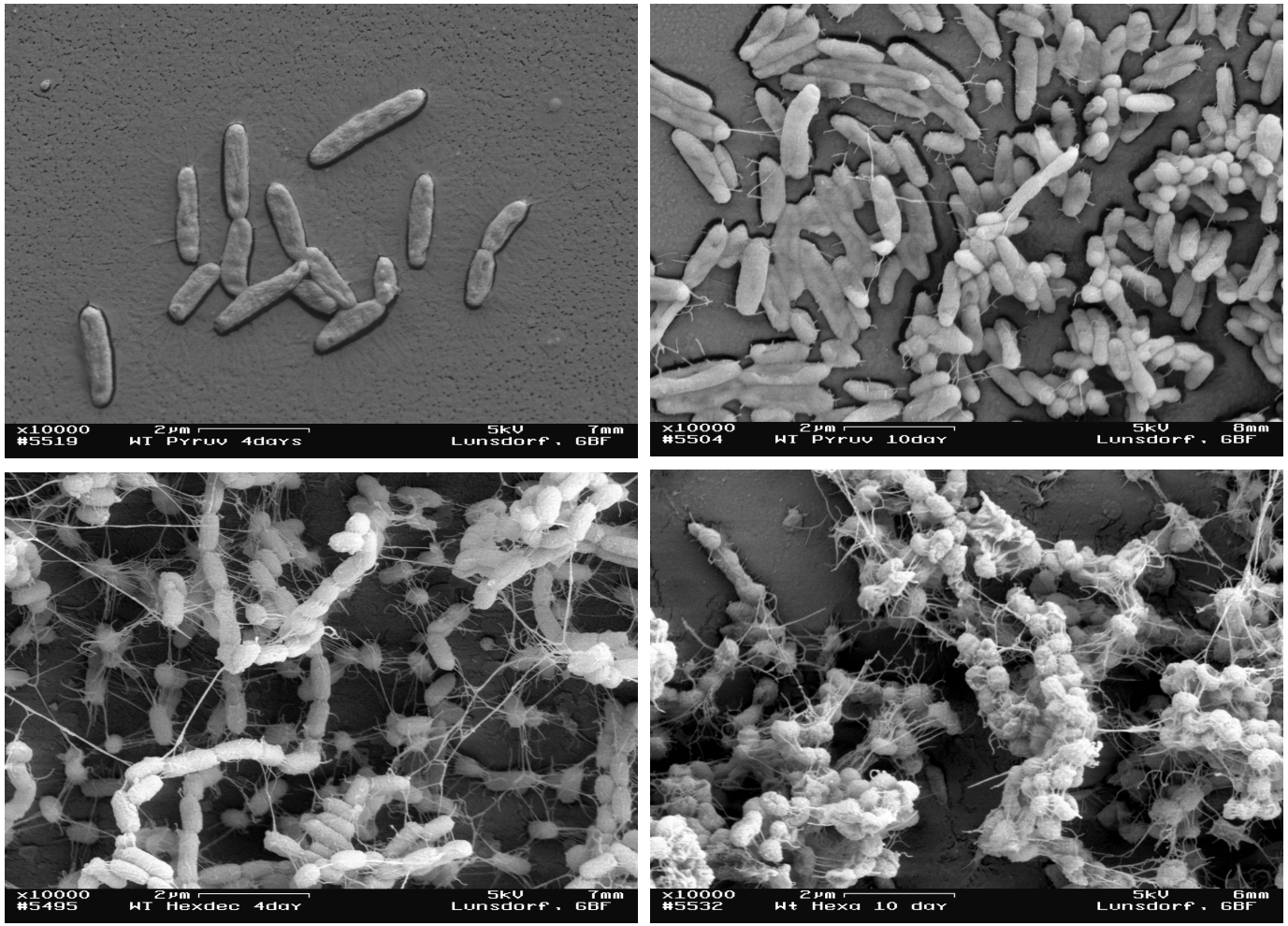


FIGURE 4. Transmission electron microscopy observations of *A.borkumensis* SK2 cells, their adhesion and biofilm formation on abiotic surfaces. The cells were grown on either pyruvate (top, left and right) or hexadecane (below, left and right) on Permanox slides in ONR7a medium. The biofilms were monitored after 4 days (left) and 10 days (right) of growth.

Aiming to characterize the genetic determimants of biofilm formation by *A.borkumensis* we employed transposon mutagenesis to identify *A.borkumensis* mutants deficient in biofilm development, as measured by the Kolter's assay, in 96-well microtitre plates made of polyvinylchloride where the attached cells are stained with cristal violet and later solibilized with ethanol and biofilm formation was measured as adsorbtion rate of cristal violet-stained cells in ethanol (Kolter and O'Toole, 1998). By applying Kolter assay, we have isolated 16 mutants deficient in biofilm formation with comparable to the wild type growth characteristics (Figure 5). For 11 of these mutants the sites of mini-Tn5 insertions were identified by inverse PCR (Table 3). Here below their analysis is presented.

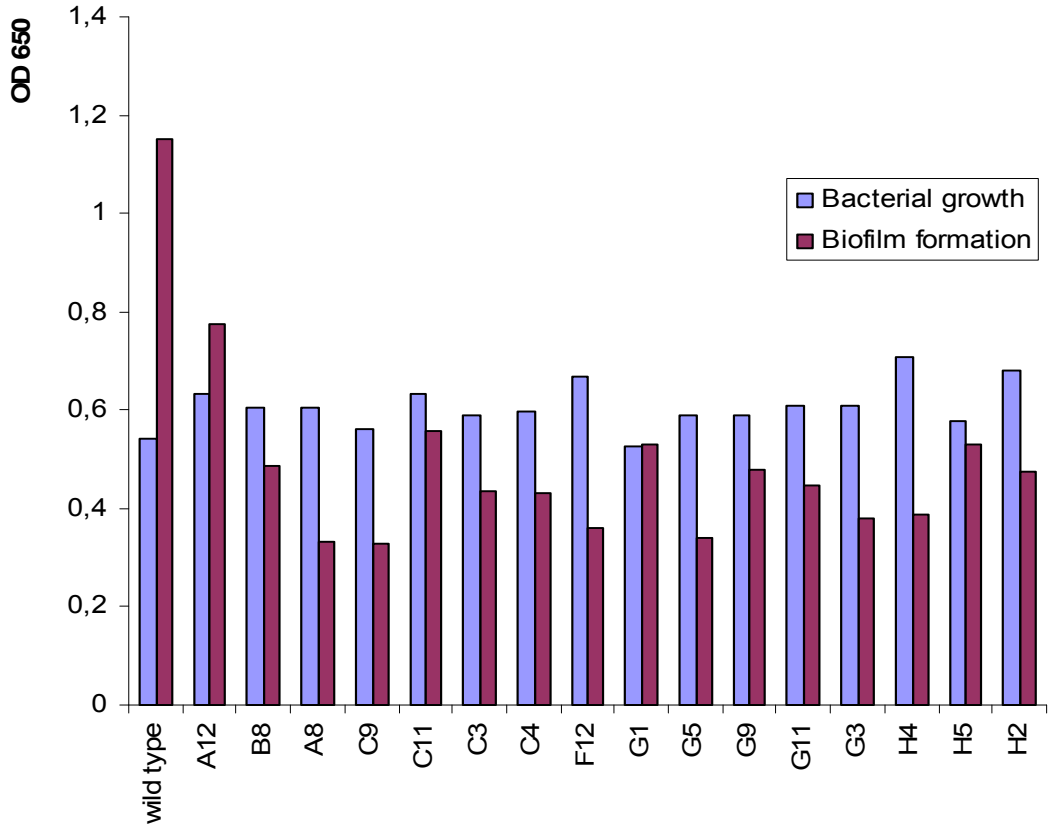


FIGURE 5. Decreased levels of biofilm formation in different mini-Tn5 mutants. The cells were grown at 30°C for 72 hours in 96-well mictotitre plates and the biofilm formation was assessed as described in Materials and Methods section. The levels of CV (crystal violet) staining are expressed relative to the final cell density measured prior to the biofilm assay, namely OD_{600} of dissolved CV versus OD_{600} of culture. The results presented here represent the mean value of two independent experiments.

B) Biofilm-deficient mutants defective in components of signal transduction systems.

Three of the biofilm-deficient transposon mutants are deficient in different proteins that are likely to be involved in signal transduction. One of them is 6M17 deficient in ABO_1986, which encodes a putative two-component sensor histidine kinase. Database searches showed that this protein exhibits significant homology over its entire sequence to signal transduction histidine kinases of various bacterial species, with highest homology to the marine gamma- and alfa-proteobacteria, including *Marinobacter aquaeolei* (VT8, 58% identity, 85% similarity), *Microbulbifer degradans* (2-40, 56% identity, 72% similatity), and *Rhodopseudomonas palustris* (HaA2, 56% identity, 71% similarity). The *Alcanivorax* protein contains 14 transmembrane domains and three domains (identified by using the SMART database) which are found in variety of signal transduction proteins in a wide range of bacteria. Residues 696 to 762 encompass a predicted HisKA domain, which is the site dimerisation and phosphorylation of the histidine kinases. This motif has been found in many bacterial sensor protein/histidine kinases (ref). Residues 809 to 902 encompass a so-called HATPase domain, found in several ATP-binding proteins (e.g. histidine kinase, DNA gyrase B, topoisomerases, heat shock protein HSP90, phytochrome-like ATPases and DNA mismatch repair proteins). Residues 942 to 1052 encompass a so-called REC domain, the site of recognition for these proteins' response regulator, proteins which are phosphorylated by the histidine kinases. Pairs of histidine kinases and their corresponding response regulator proteins constitute so-called two-component signal transduction system generally found in bacteria, but also in certain eukaryotes like protozoa, functioning to detect and respond to environmental changes (SMART). The ABO_1986 encoding the histidine kinase is likely to be in an operon together with ABO_1987 encoding a DNA-binding response regulator belonging to the LuxR family. Remarkably, the same two-component system is also involved in UV and osmoadaptation in *Alcanivorax* SK2, as was revealed by analysis of the UV and osmosensitive mutants, where the same system was picked up.

A second mutant with deficiency in signal transduction mechanisms is designated 2E3 bearing the transposon mutation in the ABO_2691, encoding a sensory box protein. Database searches showed that this protein exhibits highest homology to a putative

diguanylate cyclase/phosphodiesterase of *Pseudomonas fluorescens* (PfO-1, 43% identity, 62% similarity) and to PAS:GGDEF protein found in a number of *Shewanella* species (*Shewanella denitrificans* OS217, 40% identity, 61% similarity; *Shewanella* sp. ANA-3, 40% identity, 62% similarity; *Shewanella baltica* OS155, 40% identity, 61% similarity). SMART analysis showed it to contain 5 transmembrane domains as well as two PAS, two PAC, and DUF1 and DUF2 domains. PAS domains, here spanning the regions 276 to 343 and 401 to 468, are involved in many signalling proteins of archaea, bacteria and eucaryotes, where they are used as a signal sensor domain. Several PAS-domain proteins are known to detect their respective signal by way of an associated cofactor, often haeme, flavin, or a 4-hydroxycinnamyl chromophore. Residues 350 to 393 and 473 to 515 encompass two PAC domains, often found to be associated with PAS domains and located C-terminal to PAS domains. Their putative role is to contribute to the 3-D PAS folding. PAS-PAC domains are present in a wide range of proteins shown to be involved in light, oxygen, and redox sensing, but also in some ion channel proteins (Ponting and Aravind, 1997; Zhulin et al., 1997). Residues 515 to 687 encompass the DUF1 (GGDEF) domain, which is homologous to the adenylyl cyclase catalytic domain. This prediction correlates with the functional information available for two GGDEF-containing proteins, namely diguanylate cyclase and phosphodiesterase A of *Acetobacter xylinum*, which together regulate the turnover of cyclic diguanosine monophosphate. Several other proteins carrying this domain also carry aditional domains with functions related to the sensing of environmental signal. These include PleD, a response regulator protein involved in the ''swarmer-to-stalked'' cell transition in *Caulobacter crescentus,* and FixL, a heme-containing oxygen sensor protein. Residues 697 to 943 encompass the DUF2 (EAL) domain. This domain is found in diverse bacterial signalling proteins. It is also called EAL after its conserved residues. The EAL domain is a good candidate for a diguanylate phosphodiesterase function, thus the couterpart of the DUF1-encoded function. The domain contains many conserved acidic residues that could participate in metal binding and are lilely to form the phosphodiesterase active site.

The third mutant showing a deficiency in a signal transduction system is mutant strain 7O13, where ABO_2433, encoding a conserved hypothetical signal transduction protein is interrupted. Database searches found this protein to exhibit highest homology to PAS:GGDEF proteins of *Ralstonia* species (*Ralstonia metallidurans* CH34, 48%

identity, 66% similarity; *Ralstonia eutropha* JMP134, 47% identity, 65% similarity) and to a conserved hypothetical protein of unknown function of *Pseudomonas aeruginosa* (PAO1, 47% identity, 66% similarity). SMART analysis showed it to have a domain arrangement similar to that of the above described ABO_2691, with three PAS/PAC domains, one DUF1 (GGDEF), and one DUF2 (EAL) domain. Interestingly, BLAST analysis of the aminoacid sequence of the protein to the *Alcanivorax* genome itself picked up ABO_2691, as the closest homologue, with 28% identity and 47% similarity.

B) Biofilm-deficient mutants deficient in cellular functions other than signal transduction systems

A mutant designated as 7H4 had a transposon insertion in ABO_1028, encoding a transcription repair coupling factor mfd. MFD stands for mutation frequency decline, which denotes the rapid decrease in the frequency of certain induced nonsense suppressor mutations occuring when protein synthesis is transiently inhibited immediately after irradiation (Selby et al., 1991). Database searches found this protein to be most homologous to the transcription-repair coupling protein of *Pseudomonas* species (*Ps. aeruginosa* PAO1, 59% identity, 74% similarity; *Ps. fluorescens* PfO-1, 58% identity, 74% similarity; *Ps. syringae pv. syringae* B728a, 58% identity, 74% similarity; *Ps. putida* KT2440, 58% identity, 74% similarity). SMART analysis of this protein revealed two domains. Residues 603 to 793 encompass a so-called DEXDc domain, characteristic for this superfamily of DEAD and DEAH box helicases. DEAD helicases are involved in the unwinding of nucleic acids. Residues 829 to 913 of the ABO_1028 encoded protein encompass HELICc domain, which stands for helicase superfamily C-terminal domain. This domain is found in a wide variety of helicases and helicase related proteins. BLAST of the aminoacid sequence of the protein to the *Alcanivorax* genome itself picked up ABO_0168 encoding recG, an ATP-dependent DNA helicase, as closest homologue, with 36% identity and 55% similarity.ABO_1028 is likely to be the first gene in an operon with the downstream located ABO_1027, encoding a conserved hypothetical protein of unknown function. The pFam search of the aminoacid sequence encoded by ABO_1027 identified a GDNF (Glial-cell-line-derived neurotrophic factor) domain. This family consists of (GDNF) and neurturin (NTN) these receptors are potent survival factors for sympathetic, sensory and central nervous system neurons.

Mutant 7020 dislays a mutation in Abo_2146 which encodes glnE glutamate-ammonia ligase adenylyltransferase. GlnE is a regulatory protein which is involved in the regulation of glutamine synthetase activity (see reviews in Merrick and Edwards, 1995). Glutamine synthetase, a key enzyme in nitrogen metabolism, is responsible for the incorporation of ammonium into glutamate to produce glutamine at low ammonia concentrations. The glnE protein of *Alcanivorax* is mostly homologous to its homologues in *Pseudomonas aeruginosa* (PAO1, 53% identity, 67% similarity), and *Marinobacter aquaeolei* (VT8, 51% identity, 66% similarity). Abo_2146 is likely to be operon-arranged with the downstream located ABO_2301, encoding ilvE, a branched-chain amino acid acyltransferase.

Mutant 3G3 carries mini-Tn5 mutation in ABO_2248 encoding a putative ubiquinone biosynthesis protein. Ubiquinone is a well known component of electron transport chains. Database search shows as closest homologous proteins 2-polyprenylphenol 6-hydroxylases of *Marinobacter aquaeolei* (VT8, 45% identity, 62% similarity) and of *Shewanella amazonensis* (SB2B, 45% identity, 64% similarity). BLAST of the aminoacid sequence of the protein to the *Alcanivorax* genome itself picked up a distantly related aarF ubiquinone biosynthesis protein encoded by ABO_1192 (32% identity, 53% similarity). The ABO_2248 gene presumably forms part of an operon comprising other genes of ubiquinone biosynthesis, namely ABO_2246 encoding an ubiquinone biosynthesis methyltransferase and ABO_2247 encoding a conserved hypothetical protein.

Mutant strain 2J23 was found to be interrupted in Abo_1957, encoding a membrane-bound lytic transglycosylase B. Lytic transglycosylases cleave the *N*-acetylmuramic acid-ß-1,4-*N*-acetylglucosamine bond in peptidoglycan and catalyze the formation of a 1,6-anhydro bond on the *N*-acetylmuramic acid. *In silico* analysis of the surrounding regions showed this gene to be operon arranged with two upstream-located genes, ABO_1955 encoding a putative penicillin-binding protein 2, and ABO_1956 encoding a rod-shape-determining protein rodA, and a downstream-located gene ABO_1958 encoding rlpA lipoprotein. Such a similar cluster of genes has been studied in *E.coli,* where it was shown to be responsible for ensuring the normal cellular rod shape, but also for mecillinam sensitivity of this organism (Tamaki et al., 1980).

3.3 Production, overproduction and excretion of polyhydroxyalkanoates by *Alcanivorax* SK2 and its mini-Tn5 mutant

3.3.1 Production of polyhydroxyalkanoate by *Alcanivorax* strain SK2

For marine bacteria including Alcanivorax, oil pollution constitutes a temporary condition of carbon excess coupled with limiting nitrogen, i.e. a high carbon/nitrogen (C/N) ratio, which is precisely the condition that ordinarily favours PHA formation (Steinbüchel et al., 1991), so we would expect *Alcanivorax* to exploit PHA synthesis and storage as a means of surviving periods between oil pollution events.

In order to test by electron microscopy if *Alcanivorax* is a PHA-producer, the bacteria were grown at 30ºC in modified ONR7a medium containing 0.27 g/l of NH_4Cl and either 1.5% (w/v) octadecane or 2% (w/v) pyruvate as carbon sources (conditions of a high C/N ratio). Alternatively, conditions of low C/N ratio with either 0.15% octadecane or 0.2% pyruvate were also tested. As can be seen in Figure 6, SK2 forms large intracellular electron translucent granules, suspected to be intracellular PHA inclusions, when grown in seawater minimal medium with pyruvate (Figure 6a) and octadecane (Figure 6b) at high C/N ratios. Interestingly, granules were also formed at low C/N ratios, when the carbon source was octadecane (Figure 6d), which is metabolized by ß-oxidation: this may suggest that ß-oxidation of long chain alkanes can favour the production of storage granules.

In order to test by chemical analysis if *Alcanivorax* is a PHA-producer, the bacteria were grown at 30ºC in modified ONR7a medium containing 0.27 g/l of NH_4Cl and either 1.5% (w/v) octadecane or 2% (w/v) pyruvate as carbon sources (conditions of a high C/N ratio), the cells were recovered by centrifugation and lyophilised. Cells were treated with chloroform, and the extract was analysed by gas chromatography/mass spectroscopy analysis as described. Chemical analysis of granule-containing SK2 cells confirmed the presence of PHA polymers (Table 4), and showed it to consist of a repeat unit composition of β-hydroxy acids ranging from C_6 to C_{12}, with β-hydroxydecanoate as the dominant monomer compound. The amounts of PHA produced under the different cultivation conditions reflected what was observed in the electron micrographs, namely 3x more with alkane (18 mg/l: 14.1% of dry cell weight) than with pyruvate (6.5 mg/l: 2% of dry cell weight) as carbon source.

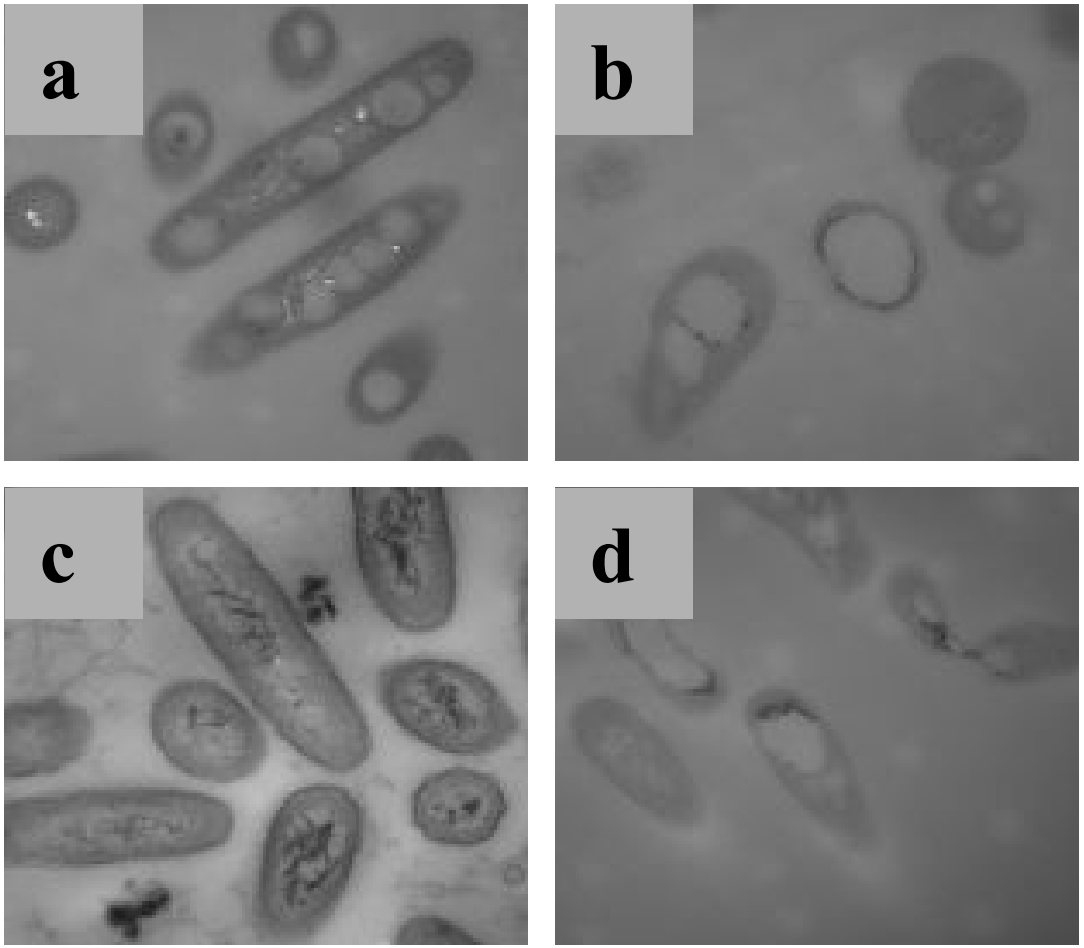


FIGURE 6. (a-d) Transmission electron micrographs of thin sections of *A. borkumensis*. Bacteria were grown in minimal seawater medium with either pyruvate **(a, c)**, or octadecane **(b, d)** as carbon source, and at C:N ratios of either 100:1 **(a, b)**, or 10:1 **(c, d)**. Storage granules are prominent in the cells in a,b,d.

3.3.2 Isolation of a mutant strain C9 showing hyper-production and excretion of PHA

In the course of the screening of a mini-Tn5 transposon library based on mini-Tn5 Str/Sp element applying Kolter's assay to look for biofilm deficient mutants, a number of mutants that failed to form a biofilm in 96-well microtitre plates were isolated (see Fig.5). Scanning electron micrographs of SK2 wild-type (Fig. 7a) and C9 mutant (Fig. 7b) cells grown on *Permanox*® hydrophobic slides covered with octadecane in ONR7a show that the mutant cells are embedded in a dense extracellular network of material, whereas the wild-type cells are not. As the cells were grown in excess of carbon source, which are conditions favouring PHA production, we suspected the extracellular polymer to be PHA. The wild type SK2 and the mutant C9 were grown on ONR7a with 2% pyruvate or 1.5% octadecane as carbon and energy sources under conditions favouring PHA accumulation (C:N ratio of 100:1), and the polymer was extracted from the total cell cultures and analysed as described above. We found that in the C9 mutant grown on octadecane, the amount of PHA was almost 2.6 g/l, which is about 23x more than that of C9 mutant cells grown on pyruvate (0.1 g/l), and 140 times that produced by the SK2 parental strain grown on octadecane (0.18 g/l) (Table 4). As no PHA was detected in the culture supernatant of SK2 wild type cells, we conclude that essentially all of the PHA produced by the SK2 wild type was intracellular. As C9 mutant cells could not properly be separated from the culture media by centrifugation (most likely due to the extracellular PHA tightly attached to them), we therefore determined PHA yields in total cell culture only.

TABLE 4. Polyhydroxyalkanoate (PHA) production in SK2 wild type and C9 mutant strains grown on different carbon sources

SK2 variant	Substrate	PHA yield (mg/l)	**Monomer composition of β-hydroxyalkanoates (mol%)**			
			C_6	C_8	C_{10}	C_{12}
SK2 wild type	Pyruvate	6.5[a]	2	14	46	28
SK2 wild type	Octadecane	18.0[a]	2	20	48	30
C9 mutant	Pyruvate	112[b]	9	15	35	41
C9 mutant	**Octadecane**	**2560[b]**	**4**	**18**	**37**	**39**

[a]β-polyhydroxyalkanoates were determined in lyophilized cells of *A. borkumensis* grown in ONR7a medium containing either 2% of pyruvate (327 mg cell dry weight per l) or 1.5% octadecane (127 mg cell dry weight/l).

[b] β-polyhydroxyalkanoates were determined in total cultures of *A. borkumensis* grown in ONR7a medium containing either 2% of pyruvate (273 mg cell dry weight per l) or 1.5% octadecane (103 mg cell dry weight/l).

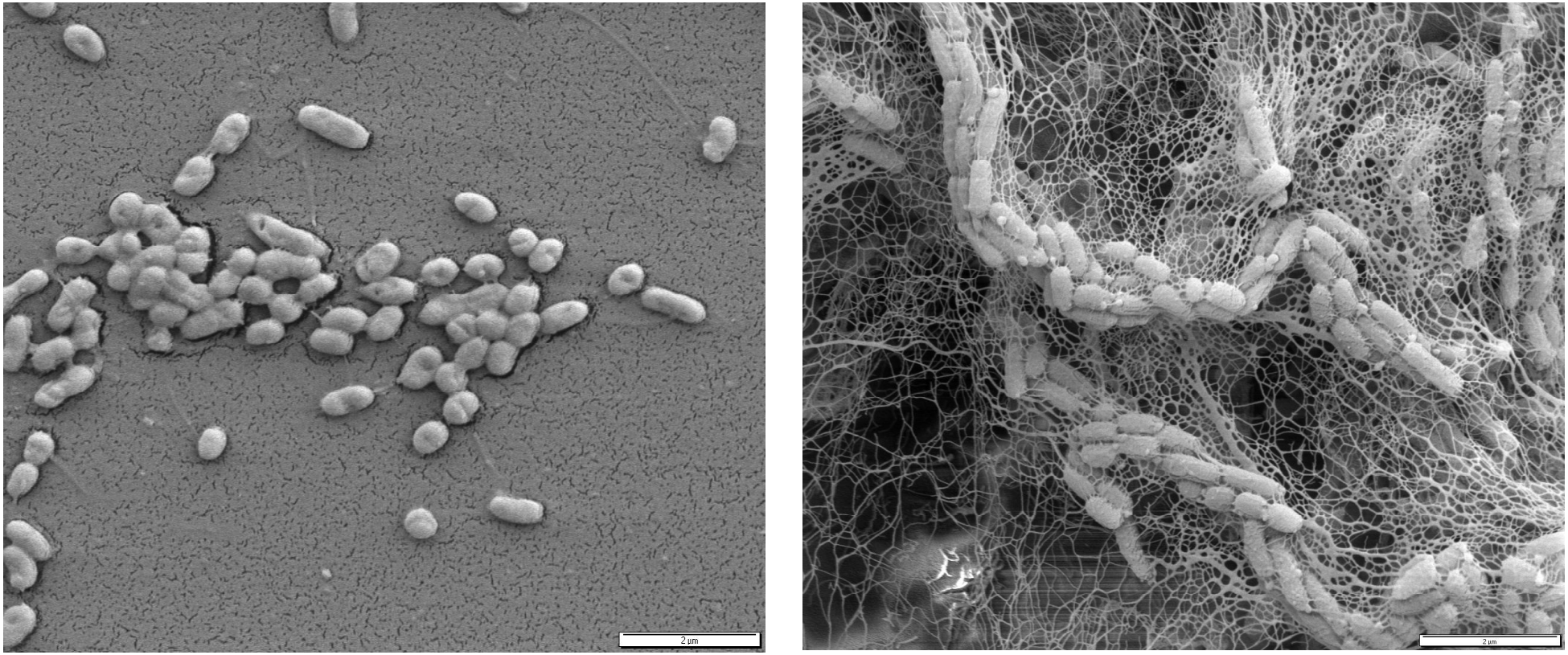
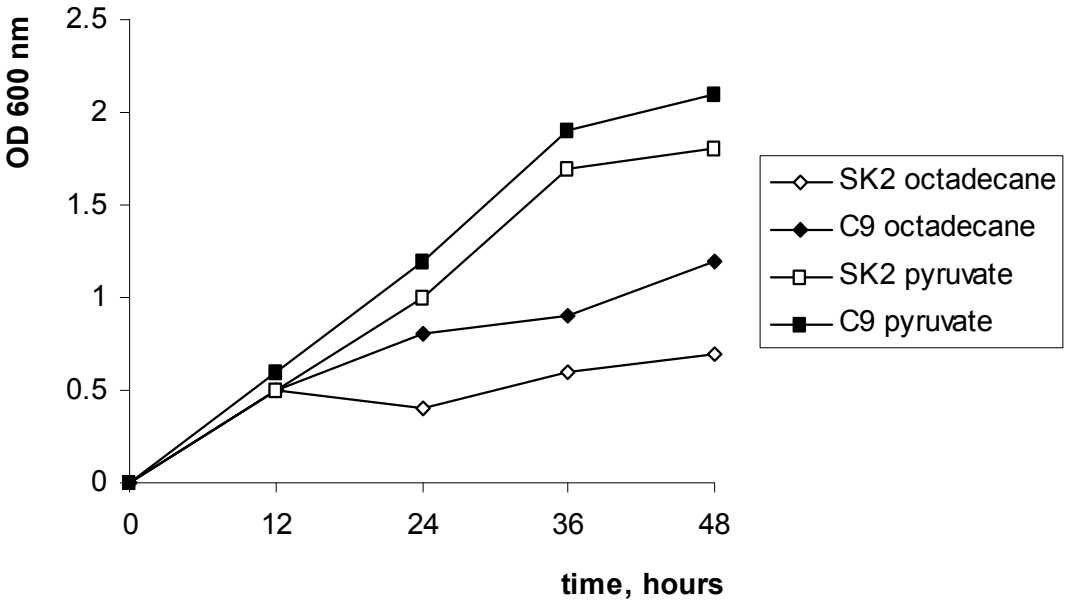


FIGURE 7. Overview of *A. borkumensis* SK2 and its mini-Tn5 mutant C9 cells by scanning electron microscopy. Scanning electron micrographs of SK2 (left) and its mini-Tn5 mutant C9 (right) whole cells grown on Permanox® slides covered with octadecane in ONR7a medium containing 1.5% (w/v) octadecane and 0.27 g/l of NH_4Cl.

3.3.3 Physiological characteristics of the C9 mutant

The hyper-production of extracellular PHA by the C9 mutant is potentially of considerable practical value and might constitute the basis of a new cell factory for PHA production that would circumvent a major hurdle to the wider utilization of PHA-based polymers. However, for this to be the case, it is essential that the mutant be robust and grow as well as the parental strain. Figure 8 shows that, at a high C:N ratio, the growth rates of the SK2 parent and the C9 mutant on pyruvate as carbon source were similar. However, on octadecane, although the initial rates were similar, they subsequently diverged, with the mutant growing faster. The absorbance measurements shown in the Figure 8 were also confirmed by viable cell counts (data not shown). The good growth of the parental strain on pyruvate when producing little PHA, its poor growth on octadecane under PHA-producing conditions, and the good growth of the mutant on octadecane under PHA-producing conditions, suggested that intracellular PHA accumulation somehow compromises cellular physiology, and that this metabolic restriction on growth is circumvented through PHA excretion.

Figure 8. The growth characteristics of *A.borkumensis* SK2 wild type and the mini-Tn5



C9 mutant grown on octadecane (a) or pyruvate (b) under the conditions of high C:N ratio.

3.3.4 Genetic basis of the C9 mutation: knockout of a tesB-like gene

The site of insertion of the mini-Tn5 element in the C9 mutant was determined by inverse PCR and was found to be located between nucleotides 557 and 558 of the CDS of the gene ABO_1111, which has been annotated as a putative TesB-like acyl-CoA thioesterase II. The site of insertion predicts a disruption of the gene's function. Since the inverse PCR reaction produced only one amplicon, we concluded that the phenotype of C9 results from the identified single transposition event. Inspection of the 3' downstream region of ABO_1111 revealed the presence of a second CDS, ABO_1112 of 645 bp in length, which overlaps the last codon of ABO_1111 (Fig. 9). The close proximity of ABO_1111 and ABO_1112 suggests that these two CDSs may form an operon. To determine whether the transposon might have a potential polar effect by interruption of the expression of a single operon's transcript, RT-PCR was used with primers Oligo I and Oligo II which specifically amplify the 311 bp region spanning ABO_1111 and ABO_1112 (Fig. 9). In both the mutant and the wild type, we obtained the expected PCR product of approximately 311 bp. This confirms that ABO_1112 is well expressed also in the C9 mutant either as a part of an operon with ABO_1111 with no polar effect of the insertion, or with ABO_1112 being transcribed from its own promoter. In any case, the amplified transcript indicative of ABO_1112 expression appears to be of comparable intensity in both SK2 and the C9 mutant.

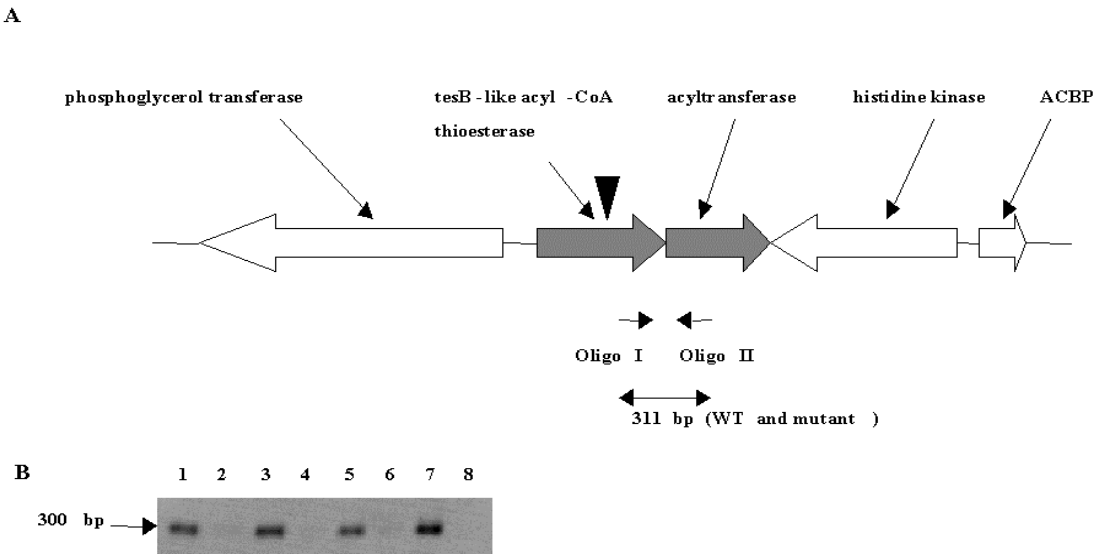


FIGURE 9. RT-PCR analysis of RNA extracted from *A.borkumensis* SK2 and tesB-like acyl-CoA thioesterase mutant. (A) Organization of the operon and adjacent genes, location of the primers used, and predicted size of RT-PCR product. (B) RT-PCR products were obtained by using total RNA extracted from WT and the mutant with the primers Oligo I and Oligo II. Lanes: 1, WT on pyruvate; 3, WT on octadecane; 5, mutant on pyruvate; 7, mutant on octadecane; lanes 2, 4, 6, 8 are corresponding negative controls (without reverse transcriptase).

3.3.5 Characterization of *A. borkumensis* tesB-like gene expressed in *E.coli*

To prove that TesB-like protein specifically cleaves hydroxyacyl-CoAs the *A.borkumensis tes*B-like gene was cloned into pCDFDuet-1 expression vector (Novagen) and expressed in E.coli RosettaBlue^{TM} DE3 competent cells (Novagen). *E.coli c*rude extracts containing the expressed TesB-like gene product were tested for enzymatic activity of the TesB-like protein. As substrates we provided acyl-CoAs (Sigma Chemicals Co., St. Louis, Missouri) and (*R,S*)-3-hydroxyacyl-CoAs *in vitro* synthesized as described (Rehm et al., 2002) and analyzed the reaction products by a 5,5-ditio-bis(2-nitrobenzoic acid) (DTNB)-assay as described (Zhuang et al., 2002) with *E. coli* harboring only vector pCDF as negative control. The data in Fig. 10a clearly demonstrate that TesB-like enzyme was able to hydrolyze efficiently hydroxylacyl-CoAs ranging from hydroxy-hexanoate to hydroxy-decanoate, with some strong preference for long-chain derivatives. By contrast, when we applied the corresponding non-hydroxylated acyl-CoAs (from hexanoyl to decanoyl) as substrates, we found that the TesB-like protein exhibits little ability to hydrolyze these acyl-CoA substrates (Fig. 10b). As *E.coli* crude extracts containing the cloned tesB-like gene displayed a high ratio of hydroxyacyl-CoA to acyl-CoA activity (approx. 500:1 for C_{10}-derivatives), we conclude that the tesB-like gene encodes a product which specifically acts on hydroxylated acyl-CoAs, and therefore can be named as hydroxyacyl-CoA-specific thioesterase.

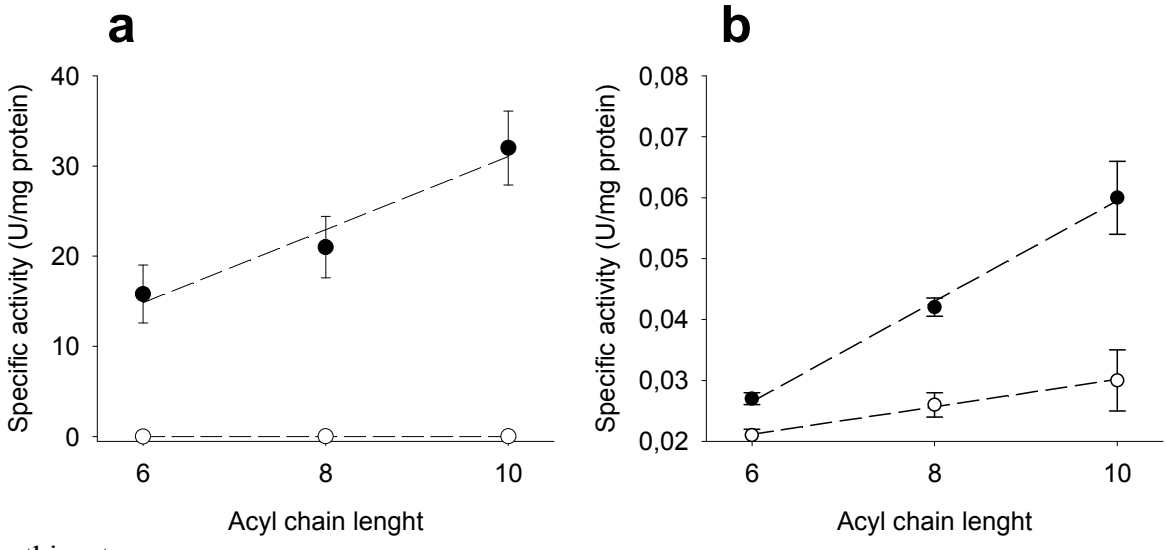


FIGURE 10. Enzyme hydrolysis of (*R,S*)-3-hydroxyacyl-CoAs **(A)** and acyl-CoAs **(B)** by crude extract of recombinant *E. coli* harboring only vector pCDF (○) or pCDFtesB-like (●). The data are means ± standard deviation of three independent samples and three independent assays. The specific activity of crude extract of *E. coli* harbouring only vector pCDF was with all substrates lower than 0.025 U/mg, which is in the range of previously published data.

To conclude, a new enzyme is reported here which specifically acts on hydroxylated acyl-CoA and that mini-Tn5 mutation in hydroxyacyl-CoA-specific thioesterase in the marine oil-degrading bacterium *Alcanivorax borkumensis* leads to the hyper-production of extracellularly deposited PHA and presents a novel system that provides high yields of the polymer which can be easily recovered from the medium. Since this system circumvents the need for costly procedures for the extraction of PHA granules from producer cells, it constitutes a promising point of departure of a novel cell factory for bioplastic production.

4 DISCUSSION

4.1. Alcanivorax in the presence of oil spill: terminal oxidation of alkanes, intracellular carbon fluxes, changes in the membrane composition and cofactor synthesis

In this study, an initial functional genome analysis to gain insight into the expression of the specific metabolic circuites characteristic of this unique marine, oligotrophic, alkane-degrading bacterium was performed to identify alkane-induced proteins. Although ubiquitously found in marine environments, *A.borkumensis* is of particular ecological interest because of its prevalence under conditions of marine oil spills (Kasai et al., 2001; Kasai et al., 2002; Syutsubo et al., 2001), with alkanes being a major component of crude oil. Proteome analysis of both the cytoplasmic and the membrane fractions of *Alcanivorax borkumensis* SK2 was performed, followed by the application of appropriate bioinformatics tools. The results of this research strongly suggest that alkane degradation in *Alcanivorax* proceeds via several alternative roots of terminal oxidation, and that it strongly affects the cells overall metabolism, including intracellular carbon fluxes, membrane lipid composition, cofactor biosynthesis and the functioning of various membrane transport systems. It also shed some light on how these particular functions are regulated.

Terminal alkane oxidation

Alkanes are generally metabolized through a primary attack of monooxygenases, which introduce oxygen into the terminal group to produce the corresponding alcohol. The subsequent steps of terminal alkane oxidation are catalyzed by alcohol and aldehyde dehydrogenases, which produce aldehyde and fatty acid correspondingly. The main enzymes catalyzing terminal alkane oxidation are usually membrane-associated (Whyte et al., 2002; Ratajczak et al., 1998; Smits et al., 2002). Analysis of our results from membrane fraction-derived spots revealed several monooxygenases and enzymes catalyzing the subsequent steps, which are uniquely expressed on hexadecane (Table 1). Among them was the entire set of enzymes of the *alkB1* operon described earlier by van Beilen et al. (2004) for *A. borkumensis* AP1, a strain variety of the same species as *A.borkumensis* SK2, comprising ABO_2707 encoding AlkB1 alkane monooxygenase, ABO_2708 encoding AlkG rubredoxin, ABO_2709 encoding AlkH aldehyde dehydrogenase, and ABO_2710 encoding AlkJ alcohol dehydrogenase (Fig.11A). We also detected alkane-induced expression of the regulator AlkS (ABO_2706), which in

divergent orientation preceeds the alkB1 operon, and which has been described to be the transcriptional activator of the alkB cluster in *Pseudomonas oleovorans* (Eggink et al., 1998). Our findings in some aspects contrast with data from the transcriptional analysis studies on *A. borkumensis* AP1 strain by van Beilen et al. (2004), who did not find alkane-induced expression of AlkS, while in the same study they found AlkB1 and another AlkB-like protein (AlkB2) to be up-regulated. Again contrasting with the transcriptional data from strain AP1, indicating enhanced expression of AlkB2, in our proteomic study on strain SK2 we could not detect such up-regulation of AlkB2 on alkanes. While strain-specific differences in regulation between strains SK2 and AP1 are unlikely, it is possible that instability of the *alk*S and *alk*B2-specific mRNAs or protein products, respectively, can account for the observed discrepancies of van Beilen's and our data.

Other enzymes found to be alkane-induced, were cytochrome P450 monooxygenase encoded by ABO_0201 (and/or ABO_2288, see below) and AlkJ2 alcohol dehydrogenase encoded by ABO_0202, which form part of a putative operon comprising the genes encoding ferredoxin (ABO_0200), cytochrome P450-1 (ABO_0201), AlkJ2-alcohol dehydrogenase (ABO_0202), and an oxidoreductase (ABO_0203) (Fig.11C). The P450-1 putative operon is closely linked to an AraC-like transcriptional regulator (ABO_0199) reading into the opposite direction. The amino acid sequence of the AlkJ2-encoded alcohol dehydrogenase (ABO_0202) shows strong homology to at least two other *A. borkumensis* alcohol dehydrogenases, one of which is AlkJ (ABO_2710) of the *alk*B1 operon. P450-1 cytochrome encoded by ABO_0201 is identical to a second P450 cytochrome, P450-2, encoded by ABO_2288 (Fig.11D), and also strongly homologous to a third P450 cytochrome, P450-3, encoded by ABO_2384 (Fig.10E). P450 cytochromes belong to a gene superfamily of heme proteins found in all eukaryotes, as well as in most prokaryotes and archaea (Nelson 1993), catalyzing the monooxygenation of a wide variety of organic molecules. A role of this enzyme in alkane degradation has already been shown for *Corynebacterium sp*., *Acinetobacter calcoaceticus* EB 104, and some hydrocarbon-degrading yeasts (Cardini and Jurtshuk, 1968; Müller et al., 1989; Lebeault et al., 1971). Our proteomic data (Fig. 1) revealed expression of P450 (either P450-1 and/or P450-2, identical proteins) on both alkane and pyruvate with clear up-regulation on alkane. Whereas P450-1, although not apparent on 2D gel, presumably is up-regulated on alkanes, since it is in the same operon with the

upregulated AlkJ2 gene, this is probably not the case for cytochromes P450-2 and -3. By performing an *in silico* comparison of the upstream regions preceeding ABO_0201 and ABO_2288 to the well-known promoter consensus sequences of other bacteria, we found that these two genes are preceded by different putative promoters and are thus likely to be differently regulated. While ABO_0201 is likely to be expressed on alkanes, ABO_2288 is probably constitutively expressed. It is not yet clear which role other than in the primary attack on alkanes cytochrome P450 may play in *Alcanivorax*, for example in our case when pyruvate is used as a carbon/energy source. There is evidence that cytochrome P450 has other metabolic roles in the cells, e.g. supplying pimelic acid equivalents for biotin biosynthesis in *Bacillus subtilis* (Cryle and Voss, 2004), with biotin being a major cofactor of the main enzymes of fatty acid biosynthesis.

We have also detected alkane-induced expression of a putative monooxygenase encoded by ABO_0190 (Fig. 11F). *In silico* analysis identified this monooxygenase as a flavin-binding monooxygenase (FMO), belonging to a whole family of xenobiotic-metabolising enzymes (Pfam). Within this family, ABO_0190 has some similarity to cyclohexanone monooxygenases (52% of identity, 68% of similarity to *Ralstonia eutropha* JMP134 protein), enzymes that mediate the oxidation of cyclohexanone, i.e. the second step of cyclohexane metabolism. An inspection of the *Alcanivorax* genes surrounding ABO_0190 revealed a putative operon of 4 genes encoding proteins presumably involved in the metabolism of cycloalkanes, starting off from cyclohexanol and finally yielding 6-hydroxyhexanoic acid, which is then presumably excreted from the cell. Thus, *in silico* analysis enabled us to assign new putative functions related to cyclohexane degradation to the found ORFs which surrounded the ABO_0190. These ORFs included caprolactone hydrolase (ABO_0191), cyclohexanone monooxygenase (ABO_0190), cyclohexanol dehydrogenase (ABO_0189) and metal-dependent hydrolase (ABO_0188). Since, we could not find a gene encoding cyclohexane monooxygenase in *Alcanivorax* genome, we suspect that in *Alcanivorax* another enzyme might mediate the initial attack of cyclic alkanes, and that this enzyme may in fact be alkane hydroxylase (encoded by either *alk*B1, or by *alk*B2). This hypothesis is based on recent findings by Fujii et al. (2004), who have shown biotransformation of cycloalkanes by the alkane hydroxylase system from *Gordonia* sp. TF6, comprising alkane 1-monooxygenase AlkB, rubredoxin AlkG, and rubredoxin AlkT. The assumption that in *Alcanivorax* the initial oxidation of cyclohexanes is mediated by the

same monooxygenase catalyzing the initial oxidation of linear alkanes, namely AlkB1 (or AlkB2), is further supported by the following notions: (i) inability of *A. borkumensis* SK2 to grow on cyclohexane as sole source of carbon and energy (data not shown), while it is well co-metabolized in the presence of crude oil (Yakimov et al., unpublished); (ii) the apparent co-induction by *n*-alkane hexadecane of the *A. borkumensis* SK2 AlkB1 and the predicted cyclohexane-degrading operon containing the up-regulated putative cyclohexanone monooxygenase (ABO_0190), which is also reflected in some marked similarities of the respective operon upstream regions both exhibiting putative σ^{70} promoters as well as some perfectly conserved sequence motives of a likely regulatory function (data not shown). However, the prediction of the function of this putative monooxygenase in *Alcanivorax* described above is highly speculative and more experimental evidences are needed to clarify the role of this enzyme in the context of alkane metabolism in *Alcanivorax*.

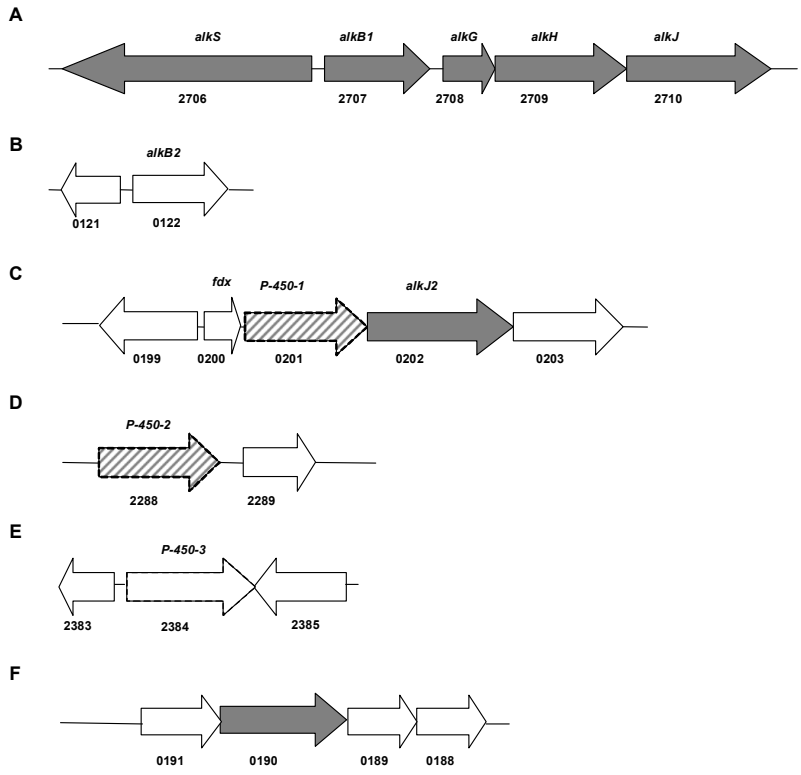


FIGURE 11. Schematic representation of genes or clusters of genes containing monooxygenase encoding genes presumably involved in terminal oxidation of alkanes, as identified by up-regulated expression on 2-DE gels (genes colored in grey). White-colored ORFs with black frame show homologous genes in the *Alcanivorax* genome, that are seemingly not up-regulated; and ORFs with upward diagonal pattern show P450-1 and P450-2 genes.

Fatty acids, lipids and membranes

Fatty acids produced during growth on alkanes are transformed into CoA-activated fatty acids which can be further degraded via β-oxidation. As found in other bacteria, our proteomics data show that alkane degradation in *A. borkumensis* goes along with an increased expression of enzymes of the β-oxidation pathway. In fact, we found the two complete sets of enzymes of the β-oxidation pathway to be induced by alkanes: we detected alkane-induced expression of ABO_0184 and of ABO_2748 both encoding fatty acid CoA ligases (or synthetases), ABO_2102 and Abo_0988, both encoding acyl-CoA dehydrogenases, ABO_1652 and ABO_1566, both encoding bifunctional components of the β-oxidation multifunctional enzyme complex and that posseses both 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities. ABO_1652 and ABO_1566 form part of two different enzyme complexes involved in alkane-induced fatty acid oxidation, one of which, ABO_1652 corresponding to fadB2 of the fadAB2 operon, is exclusively expressed in cells grown on hexadecane, while the latter, ABO_1566 corresponding to *fad*B of the *fad*AB operon, though clearly up-regulated in presence of alkanes, is also expressed in cells grown on pyruvate.

Apart from enhanced expression of genes mediating fatty acid degradation, we also detected, at first sight unexpectedly, a significant alkane-induced increase of expression of fatty acid biosynthetic genes. Thus, we found alkane-induced up-expression of the *fab*AB operon (ABO_0835 and ABO_0834) encoding beta-hydroxyacyl-acyl carrier protein dehydratase (FabA) and beta-ketoacyl-acyl carrier protein synthase I (FabB). In addition, up-expression of a second FabB homologue, encoded by ABO_1520 was also detected on alkanes. These two enzymes encoded by the *fab*A and *fab*B genes, respectively, are, however, specifically required for the synthesis of unsaturated fatty acids only, as it was shown for *E. coli* (Silbert and Vagelos, 1967; Cronan et al., 1969) and *Pseudomonas aeruginosa* (Hoang and Schweizer, 1997). Accumulation specifically of unsaturated fatty acids during exposure to *n*-alkanes, leading to an increase of lipids with unsaturated fatty acids in the membrane, has been reported for *Pseudomonas oleovorans* GPo1 (Chen et al., 1995), where it has been shown that induced expression of *alk*B alkane hydroxylase during growth on alkanes leads to abundance of this inner membrane protein (Eggink et al., 1987) and to its incorporation into the membrane, thereby affecting its structure and physical characteristics. It is believed that lipids with

unsaturated FAs can contribute to keep the membrane fluid despite the large fractions of AlkB proteins in the membrane.

We furthermore detected alkane-induced down-regulation of cytoplasmic proteins acetyl-CoA carboxylase AccA encoded by ABO_1159 and acetyl-CoA carboxylase AccC encoded by ABO_2010, components of a multicomponent system, which is required for fatty acid biosynthesis. Down-regulation of these two genes catalyzing the first reaction in biosynthesis of fatty acids, which is the production of malonyl-CoA from acetyl-CoA, can be explained by a reduced requirement of alkane-grown cells for malate, which is produced in abundance through increased activity of the glyoxylate cycle during growth on alkanes (see below).

Alkane-induced changes of the composition of the cellular fatty acid pool are indicative for concomitant major changes in membrane lipids composition and the membrane as a whole. In this context we noted increased biosynthesis of certain types of lipids, in particular cardiolipin. Thus, the enzyme known to be required for the biosynthesis of cardiolipin was found to be up-expressed during growth on alkanes (ABO_1816 encoding cardiolipin synthase cls). Cardiolipin has the potential to form nonbilayer structures, which introduce discontinuity into the bilayer membrane structures and thus allow for dynamic membrane functions, such as membrane fusion events e.g. during cell division and the formation of adhesion sites between the outer and inner membranes (11), but also to activate membrane-bound enzymes, like e.g. alkB (Jensen and Schutzbach, 1988; Navarro et al., 1984). Importantly, cardiolipin also may represent a protective membrane adaptation to decrease permeability of the membrane for organic solvents, as it was shown for pseudomonads (von Wallbrunn et al., 2002).

As another alkane-induced change related to the cellular lipid metabolism, we found up-regulation of several genes coding for the lipoprotein releasing Lol system (LolABCDE), which anchors lipoproteins to the periplasmic surface of either the inner or the outer membrane, depending on the sorting signal (Tokuda and Matsyama, 2004). An ATP-binding cassette transporter, encoded by *lol*CDE, transports outer membrane-specific lipoproteins at first across the inner membrane into the intermembrane periplasmic space, where a intermembrane shuttle complex is formed between the released lipoproteins and a specific periplasmic chaperone LolA. This complex then

interacts with an outer membrane-located permease LolB to pass on the lipoproteins the outer membrane (Tokuda and Matsyama, 2004). We observed alkane-induced expression of the LolCDE transporter, encoded by ABO_1049 and ABO_1050, and of the LolB permease encoded by Abo_0520. As to the periplasmic chaperone LolA encoded by ABO_1291, by contrast expression was only detected on pyruvate, a finding that is likely to be an artifact as Lol system active during growth on alkanes will result in the chaperone LolA being complexed with its target lipoproteins, and therefore present in a stage that cannot easily be resolved by proteomics. Alkane-induced expression of the Lol system in *Alcanivorax* could be linked to an increased need to release lipoproteins, as many of them have been shown to contain emulsifying properties, helping to make highly hydrophobic substrates such as alkanes accessible for microorganism (Kuiper et al., 2004; Lindum et al., 1998; Yakimov et al., 1995).

Glyoxylate bypass and gluconeogenesis

During growth on alkanes as sole carbon source, bacteria in principle have to generate all cellular precursor metabolites from acetyl-CoA, the main intermediary product from alkane degradation via β-oxidation of fatty acids. To compensate for this inability to synthesize key cellular metabolites like the 3-carbon compound phosphoenolpyruvate (PEP), directly from alkanes, the CO_2-releasing steps of the citric acid cycle are bypassed by two supplementary enzymes, isocitrate lyase and malate synthase, to form the so-called glyoxylate bypass. Therefore, not surprisingly, one of the major cytoplasmic responses to alkane exposure in *Alcanivorax* SK2 was found to be the induction of the enzymes of glyoxylate bypass (ABO_ 2741 encoding isocitrate lyase AceA, and ABO_1267 encoding malate synthase GlcB) along with the downregulation of the enzymes indicative for the complete TCA cycle including its CO_2-releasing steps (ABO_1281 encoding isocitrate dehydrogenase Icd, ABO_1494 encoding 2-oxoglutarate dehydrogenase LpdG). We also suspect alkane-induced down-regulation of another enzyme indicative for the complete TCA, namely succinyl-CoA synthetase SucC encoded by ABO_1493, as it is arranged in the same putative operon with the upstream ABO_1494, according to our *in silico* analysis.

Those enzymes of the TCA cycle that are still needed for the active TCA/glyoxylate bypass, were found to be upregulated on alkanes, namely malate dehydrogenase (ABO_1248) and succinate dehydrogenase SdhD (ABO_1499). The notion that in

alkane-growing conditions in principle all biosynthetic precursors come from acetyl-CoA, also explains why enzymes involved in gluconeogenesis, namely malic enzyme MaeB (ABO_2239) and phosphoenolpyruvate synthase PspA-1 (ABO_1427), were found to be upregulated on alkanes. As a general observation, employment of the TCA/glyoxylate bypass results in malate becoming a key cellular intermediate during growth on alkanes.

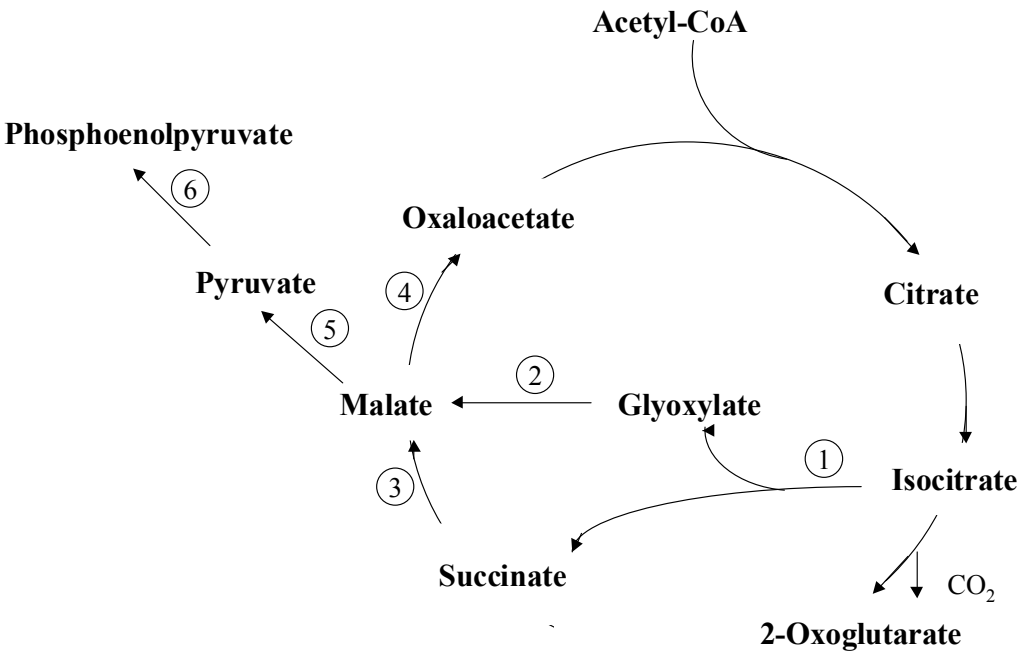


FIGURE 12. The alkane induced the ‘‘horseshoe‘‘of an incomplete TCA cycle shortcutted by glyoxylate bypass and gluconeogenesis and the corresponding alkane-induced enzymes of *A.borkumensis* SK2 (marked by numbers). The incomplete TCA cycle is associated with the alkane-induced downregulation of 2-oxoglutarate dehydrogenase. The glyoxylate bypass is carried out by isocitrate lyase (1) and malate synthase (2). Succinate produced via glyoxylate bypass is converted to malate by succinate dehydrogenase (3). Malate is converted to either oxaloacetate by malate dehydrogenase (4), or is used by malic enzyme (5) in gluconeogenesis to produce pyruvate. Pyruvate is then converted by phosphoenolpyruvate synthase (6) to produce phosphoenolpyruvate.

Polyhydroxyalkanoate biosynthesis

Growth on alkanes signifies for *Alcanivorax*, an oligotroph marine bacterium, functioning under conditions of excess of carbon, as signalled by a high C:N ratio. Consequently, growth on alkanes was thus found to favour expression of enzymes responsible for biosynthesis of polyhydroxyalkanoate, a cellular storage compound

found in many bacteria including *Alcanivorax*. One of two *A. borkumensis* polyhydroxyalkanoate synthase genes *pha*C found in the A. *borkumensis* genome (ABO_1418) was found to be solely expressed in alkane-grown cells, while the other *pha*C-encoded enzyme (ABO_2214) was not expressed during growth on alkanes. Taking into account that we found that the bacterium also produces PHA on other non-alkane substrates, when these are present under conditions of high C:N ratio (Sabirova et al., submitted for publication), we suggest that this second PHA synthase (ABO_2214) may be responsible for production of PHAs in non-alkane-grown cells under appropriate conditions. The two enzymes are likely to have different substrate profiles with respect to pyruvate- and alkane-derived precursors.

Cofactor synthesis

Alkane metabolism in *Alcanivorax* seems to impose a strong cellular demand for cofactor synthesis, as many of the respectingly active enzymes, in particular monooxygenases involved in alkane degradation, contain cofactors as active groups. Thus, we have detected the alkane-induced expression of Rib*D* ((S)-2-hydroxy-fatty-acid dehydrogenase ABO_2174), a key enzyme of the riboflavin synthesis pathway. This molecule is the precursor for flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), typical cofactors for enzymes involved in reduction processes and for electron transport proteins, such as dehydrogenases, oxidases, and monooxygenases. In our case riboflavins play a major role as cofactors of the flavin-binding monooxygenase ABO_0190 and of the FMN-binding domains of cytochromes P450 encodes by ABO_0201 and ABO_2288 genes. On the other hand, we have observed alkane-induced down-regulation of an enzyme involved in biotin biosynthesis, such as ABO_1963 encoding for lipoil-(acyl-carrier protein)-protein-*n*-lipoyltransferase. The down-regulation of biotin biosynthesis may be connected with the alkane-induced repression of the *acc*A and accC genes, encoding key enzymes of the fatty acid biosynthetic root, which need biotin as a cofactor.

4.2 *Alcanivorax* in its marine environment: adaptation to UV exposure, salt, low temperature, and biofilm formation

This study also explored the main genetic determinants of some major environmental adaptations of the marine oil-degrading bacterium *Alcanivorax* SK2 to UV radiation, salinity, low temperature as well as biofilm formation. By using transposon mutagenesis 48 mutants were isolated and were deficient in one of the four above-mentioned environmental responses.

UV adaptation

By analysing the genes interrupted by the transposons giving rise to UV-sensitive mini-Tn5 mutants of *A.borkumensis*, two major metabolic targets for UV induced cellular damage were unveiled: DNA metabolism, and the cellular redox state, disturbances of which apparently need to be repaired immediately. Thus, DNA damage requires expression of well-described DNA repair genes. Such DNA repair genes, found to be affected in UV-sensitive mutants, could be divided into three groups based on their respective molecular function. The most abundant group of mutants exhibited mutations in different components of the so-called ABC-excision repair system. Excision repair is known from other bacteria to be an UV-induced immediate response to repair DNA damage. Nucleotide excision repair proceeds DNA from both sides of the damaged nucleotide, and then the single-stranded gap is filled in and sealed (Sancar and Rupp, 1983). A second group of genes confering UV tolerance and UV sensitivity if mutated is represented by the recA protein, which initiates a so-called SOS-response, mounted by the cells when single-stranded DNA appears after exposure to radiation. The main task of the proteins of SOS response is to perform DNA repair and cell's survival after DNA damage (Au et al., 2005). One UV sensitive mutant was found to be deficient in ruvB protein, which is also known to be involved in DNA repair and recombination. RuvB probably belongs to the SOS response system in *Alcanivorax*, as it was shown for *E.coli* (Shinagawa et al., 1988; Shurvinton and Lloyd, 1982). Another gene product involved in DNA replication and seems to belong to the SOS system is rep helicase. Rep is a non-essential helicase, and has been shown to be able to remove proteins from the DNA in vitro (Yancey-Wrona and Matson, 1992), thus presumably allowing the progression of replication forks by dislodging proteins from the path of the replication machinery (Matson et al., 1994). Finally, mismatch repair seems to be also crucial in repairing DNA damages caused by UV stress in *Alcanivorax*, with the adenine-specific

DNA-methyltransefrase performing methylation of DNA for strand discrimination during replication-associated DNA mismatch repair, as it was earlier shown for *E.coli* (Marinus 1996).

In addition to the cellular nucleic acid metabolism being affected by UV, *Alcanivorax* also seems to have developed specific strategies to protect itself against oxidative stress as a direct consequence of UV radiation. Thus, several of the UV sensitive Tn5 mutants were found to be deficient in certain components of the respiratory chain, like nitrate reductase, cytochrome b561, and an iron-sulfur protein. Nitrate reductase has also been shown to be completely inactivated by UV in other species (Brito and Durourdieu, 1987), and it was also shown that menaquinone is the UV sensitive component of the electron transport chain catalysed by nitrate reductase (Brito et al., 1995). Interestingly, in *Alcanivorax* the gene encoding nitrate reductase is followed by a gene highly homologous to alkyl hydroxyperoxide reductase. In *Alcanivorax* these two gene functions are presumably tightly coupled under UV stress.

Cytochrome b561 was shown to protect against oxidative stress and to contribute to the limited dioxygen tolerance in *Moorella thermoacetica* (Das et al., 2005). The conserved hypothetical signal transduction protein encoded by ABO_2214 is presumably also involved in defence mechanisms against reactive oxygen species, as it is followed by the genes ahpC and aphD encoding different components of hydroxyperoxide reductase. The genes located downstream from another two-component sensor system consisting of pfeS and pfeR (ABO_1690 and ABO_1691) also hint at a role of this sensor system in oxidative stress response. Thus, downstream from pfeS we found genes required for iron acquisition by synthesizing and secreting iron chelators, termed siderophores, which bind iron and deliver it to the bacterial cell via cell surface receptors (Neilands, 1981). A pronounced cellular need for iron by *Alcanivorax* may be associated with an enhanced need for heme, a major component of some electron transport systems, to protect the cell against oxidative stress. UV-induced accumulation of iron seems to be a widespread mechanism among marine bacteria: induction of several iron-sequestering protein-encoding genes upon UV stress was also shown for *Shewanella oneidensis* MR-1 (Qiu et al., 2005); accumulation of metals is also thought to be the main adaptation mechanism to UV in the highly UV resistant bacterium Deinococcus radiodurans (Daly et al., 2004). However, in contrast to iron-accumulating Shewanella oneidensis and

Alcanivorax species, *Deinococcus radiodurans* accumulates very high intracellular levels of manganese and only low levels of iron. Mn(II) ions concenrated in Deinococcus radiodurans might serve as antioxidants that reinforce enzymaic systems which protect against oxidative stress during recovery from radiation exposure (Ghosal et al., 2005).

Adaptation to UV exposure is dependent on the function of different proteins involved in signal transduction, like pfeS kinase sensor protein (ABO_1690), a DNA-binding response regulator of LusR family (ABO_1987), and conserved hypothetical signal transduction protein (ABO_2433). The latter two systems also seem to play an important role in biofilm formation of Alcanivorax, representing a link between formation of a biofilm and Uvadaptation in this organism. If the first two proteins show domain arrangements typical for histidine kinases, the latter dispays a rather complex domain arrangement with three PAC, three PAS, one GGDEF and one EAL domains. PAS domains are important signalling modules that monitor changes in light intensity, redox potential, oxygen tension, the presence of small effector ligands, and the overall energy level of a cell (Taylor and Zhulin 1999). GGDEF and EAL domains are known to be involved in the metabolism of the secondary messenger diguanylate (c-diGMP). These domains seem to play an essential role not only in the adaptation of *Alcanivorax* to UV stress, but also in this organism's ability to form a biofilm, since the same signal transduction system has been identified to be affected in a mutant deficient in biofilm formation. Moreover, Tn5-mutant analysis of *Alcanivorax* identified another protein to be essential for biofilm formation by *Alcanivorax*, namely a sensory box protein, which has a domain arrangement similar to that of ABO_2433-encoded protein, with two PAS, two PAC, one GGDEF and one EAL domains. This interlinkage between two different environmental adaptations (UV and biofilm) reflected on both the genetic level (the same genes), and on the level of domain architecture (identical domain arrangement) means that cellular stress by UV and/or oxidative stress maybe coupled to biofilm formation by changes in the cellular c-di-GMP levels and/or redox potential.

Among the pathways, that, when affected by Tn5 mutations contribute to increased cellular sensitivity, were also two different transport systems. One of them encodes an ABC transporter of unknown function. The second one is TRAP transporter protein, that is involved in the transport of dicarboxylate compounds in other bacteria (Janausch et

al., 2002). For TRAP transporters the driving force for solute intracellular accumulation is an electrochemical ion gradient across the membrane rather than ATP hydrolysis (Kelly and Thomas, 2001). Therefore its function related to UV stress might be of compensatory fashion: the extensive electrochemical ion gradient generated via functioning of different electron transport proteins (see above) involved in defence mechanisms against the oxidative stress is recharged via uptake of carbon compounds across the membrane.

Production of extracellular polysaccharides is thought to provide an additional protection mechanism employed by bacteria to cope with UV stress. Thus, one UV-sensitive mutant was deficient in ABO_0908 encoding capsular polysaccharide biosynthesis protein WbpO. Biosynthesis of extracellular polysaccharides was also shown to be induced by UV-B radiation in terrestrial cyanobacterium Nostoc commune (Ehling-Schulz et al., 1997).

Adaptation to salt

Adaptation to osmotic up-shifts in *Alcanivorax* seems to proceed via the mechanisms of osmoadaptation already described for other bacteria. Thus, biosynthesis of osmoprotector ectoin has been shown to be essential for osmotic up-shifts, as one of the osmosensitive transposon mutants was decicient in EctB gene, encoding 2,4-diaminobutyrate aminotransferase, known to catalyse the second step in the biosynthesis pathway of ectoin (Peters et al., 1990; Tao et al., 1992). Biosynthesis of ectoin in response to osmotic shock was also shown for a number of microorganisms, including marine bacteria. (Talibart et al., 1994; Calderon et al., 2004 ; Kuhlmann and Bremer, 2002). It is also not unexpected, that several efflux pumps were picked up by transposon mutagenesis as relevant for osmoadaptation: ABC export system, ABC efflux transporter, and MATE efflux family protein. RNA degradation during osmotic up-shifts seem to represent one more mechanism of osmoadapation in *Alcanivorax*, as a mutation in the gene encoding polynucleotide phosphorylase was found to cause osmosensitivity in one of the transposon mutants.

Adaptation to low temperature

Adaptation to low temperature conditions in *Alcanivorax* seems to proceed via various regulatory mechanisms. One of the elements that seem to be important for low temperature adapation in *Alcanivorax* is the BipA regulator. This regulator has been

implicated in regulation of pathogenicity of enteropathogenic *E.coli* bacteria. Later it was shown to also be required for growth of *E.coli* at low temperature (Pfennig and Flower, 2001). Recently new data appeared which clarified its multifold regulatory roles: it functions as a translation regulator required specifically for expression of the transcriptional modulator Fis (Owens et al., 2004): induction of BipA triggers efficient expression of Fis, thereby modulating a range of Fis-dependent downstream processes, including coping with temperature downshift (Kim et al., 2004).
Another putative transcriptional regulator picked up by Tn5 mutagenesis as being relevant for low temperatue adaptation is encoded by ABO_1835 and belongs to the MerR family. The proteins of th MerR superfamily of transcriptional regulators promote transcription of various stress regulons. Protein-pII uridylyltransferase glnD (ABO_1141), known to belong to the nitrogen regulation system, regulating ammonia assimilation through modulating glutamine and glutamate synthetase activities, in addition, at least in *Alcanivorax*, is also involved in the adaptation of this bacterium to low temperatures.

In order to survive at cold temperatures bacteria have to be able to maintain the cell membranes in a liquid-crystalline state (Feller and Gerday, 2003). This requires temperature-dependent shifts in the membrane's lipid composition, thus making maintained membrane fluidity a crucial cellular adaptation to cold stress. Accordingly, screening for cold-sensitive Alcanivorax transposon mutants resulted in the isolation of an mmsA mutant with mmsA encoding methylmalonate semialdehyde dehydrogenase involved in the generation of isoleucine and valine degradation intermediats, which feed into the production of branched-chain fatty acids predicted to be required for corresponding lipids to maintain appropriate membrane fluidity at low temperatures. Branched-chain fatty acids have also been shown to be involved in cold adaptation in *Listeria monocytogenes* (Annous et al., 1997) and *Bacillus subtilis* (Kaan et al., 2002).

RNA decay and maturation during temperature downshift seem to represent another mechanism involved in cold adaptation in *Alcanivorax*. Thus, amongst the cold-sensitive Tn5 mutants, a mutation in the gene encoding ribonuclease D was found to affect the organism's adaptation to cold stress. Cold stress-induced RNA decay may determine drastic stabilization of the cold-shock transcripts and cold shock-induced modifications of the translational apparatus determine their preferential translation in

the cold. This preferential translation at low temperature by a modified translation mashinery targets in particular some cold-shock mRNAs (Gualerzi et al., 2003), which display specific cis elements present in the 5' untraslated region. Moreover, cold-shock induced mRNA degradation also results in increased cellular nucleoside diphosphate pools, which in turn, would trigger an increased rate of DNA replication.

Finally, a number of other genes of unknown function also seems to be implicated in adaptation to cold stress, including two conserved hypothetical proteins (ABO_2346 and ABO_1926) and a putative membrane protein (ABO_0666). They presumably represent novel mechanisms of cold adaptation and will be subject of future studies.

Biofilm formation

Biofilm formation in Alcanivorax seems to be highly dependent on the function of various signal transduction systems as it has been shown by transposon mutagenesis, where inactivation of these systems lead to deficiencies in biofilm formation. Thus, at least three different signal transduction systems (ABO_1986, ABO_2433, and ABO_2691) were shown to be implicated in the biofilm formation. The first one, ABO_1986 encoding two-component sensor histidine kinase protein, was also shown to involved in UV and osmo-adaptation. While ABO_1986 encodes a typical histidine kinase sensor protein of a two-component regulatory system, the latter two identified genes both contain dinstinct GGDEF, EAL, and PAS domains. While the GGDEF and EAL domains have previously been suggested to be involved in regulating cell surface adhesiveness of bacteria, PAS domains seem to primarily act as sensors for light and oxygen in signal transduction (Taylor and Zhulin, 1999), and therefore had not yet been found to be of relevance for biofilm formation. It maybe suspected that the PAS domains of the conserved hypothetical signal transduction protein encoded by ABO_2433 and of the sensory box protein encoded by ABO_2691 play an important role in sensing perturbations of oxygen tensions in the different layers of a biofilm. One could reason these same lines to explain the role of ubiquinone biosynthetic gene (ABO_2248), also found to lead to biofilm deficiency when interrupted by Tn5 element. A knockout mutation of the homologous ubiquinone biosynthetic gene in *E.coli* resulted not only in the inability to synthesize ubiquinone, but also severely diminished growth yields under aerobic conditions, but not so during anaerobic growth with either nitrate

or fumarate as terminal electron acceptors (Soballe and Poole 1998). These results hint at important role of the quinone pool with respect to signaling the respiratory status of bacterial cell, of particular relevance when growing as biofilm.

Unexpectedly, biofilm formation seems to also be linked to DNA repair, as evidenced by one Tn5 mutant: Mfd encoded by ABO_1028 is implicated in preferential gene repair, i.e. repair of certain regions, structures or sequences of the genome, or of only one of the two strands of the duplex at a faster rate as compared with the rest of the genome (Bohr et al., 1985). If bacteria grow as biofilm on aqueous surfaces, then they are probably more exposed and sensitive to UV radiation, than planctonic cells. Interestignly, it was shown for *S. enterica*, that bacterial biofilms although more resistant to antimicrobial substances, render their component cells more sensitive to radiation than it is the case for planctonic cells (Niemira and Solomon 2005). Therefore, mechanisms protecting the cells from UV such as DNA repair systems may play a crucial role for biofilm formation by repairing for marine bacteria UV-induced cell damages inherent with bacterial growth on surfaces exposed to UV radiation and may probably already be active at low doses of solar radiation.

The role of multidrug transporter in biofilm formation in *Alcanivorax* as suggested by Tn5 mutants carrying the transposon in ABO_2631, is not easily explained, except under the asumption that such biofilms play a role during growth on other cellular organisms. Thus it has recently been reported that a toxin-responsive efflux pump could be important for interaction of a fungal pathogen with rice plants (Urban et al., 1999). Also, multidrug resistance has been associated with bacteria adhesion to eucaryotic cells ((Di Martino et al., 1997).

4.3 A rational for hyper-production and excretion of PHA in the C9 mutant

In one of the Tn5-induced biofilm deficient mutants, strain C9, biofilm formation was found to be disrupted by large quantities of excreted exracellular material, macroscopically manifested as slime in the culture media. ABO_1111 interrupted by the Tn5 transposon was identified as the genetic basis of the observed phenotype. Amino acid similarity search of ABO_1111 against the entire *A. borkumensis* genome identified an acyl-CoA thioesterase II protein, encoded by the *tesB* gene (ABO_1111), as closest homologue. Acyl-CoA thioesterases have been mainly studied in *E. coli*, which possesses two such enzymes: (i) acyl-CoA thioesterase I (encoded by the *tesA* gene), that is specific for hydrolysis of C_{12}-C_{18} acyl-CoA esters (Bonner and Bloch, 1972); and (ii) acyl-CoA thioesterase II (encoded by the *tesB* gene), that cleaves C_6-C_{18} acyl-CoA esters as well as C_{12}-C_{18} 3-hydroxyacyl-CoA esters (Barnes et al., 1970). If TesA has been implicated in the hydrolysis of the thioester bond of acyl-ACPs generated during *de novo* fatty acid synthesis, thus generating free fatty acids for various cellular functions, or, if in excess, to be channeled into the ß-oxidation pathway in *E. coli* (Klinke et al., 1999), little is known about the physiological role of *tesB* in the bacterial metabolism; however, recent results suggest that tesB-encoded acyl-CoA thioesterase II plays an important role in 3-hydroxyalkanoic acid (3-HAA) production by cleaving 3-hydroxyacyl-CoA thioester bonds to produce free 3-HAA (Zheng et al., 2004).

The finding that in *A.borkumensis* genome apart from a tesA gene homologous to that found in E.coli both tesB and tesB-like genes were present prompted a search for CDSs of proteins homologous to the *A.borkumensis* TesB and TesB-like proteins in other bacterial species. BlastP searches identified in a number of closely related Gammaproteobacteria (i.e. *Pseudomonas* spp., *Idiomarina loihiensis*, *Acinetobacter* sp., and *Caulobacter crescentus*), all of which have been reported to produce PHA (with the exception of *Idiomarina loihiensis*, where it has not yet been tested) both tesB and tesB-like genes, with the latter CDS annotated as TesB-like thioesterase, putative acyl-CoA thioesterase II, or as hypothetical protein. The TesB acyl-CoA thioesterase II of PHA-producing *Rhodobacter sphaeroides* is, unlike the homologous acyl-CoA thioesterase II of E.coli , not able to hydrolyse 3-hydroxyacyl-CoA substrates (Seay and Lueking, 1986), hinting at different substrate specificities of the various acyl-CoA thioesterase homologs, so the existence of both a tesB and a second *tesB*-like gene in *Alcanivorax*

and other PHA producing bacteria may reflect distinct functions of TesB and TesB-like proteins, with the TesB product acting on acyl-CoA derivatives whereas the TesB-like protein specifically cleaves hydroxyacyl-CoAs. A plausible explanation for the phenotype of the C9 mutant would therefore seem to be that the mutation inactivates a tesB-like gene, abolishes release of free 3-HAA from 3-HAA-CoA, and thus increases the pool of the PHA precursor 3-HAA -CoA, resulting in enhanced formation of PHA (and its subsequent excretion; see below). The potential metabolic circuits relevant to this scenario in *Alcanivorax* are depicted in Figure 13.

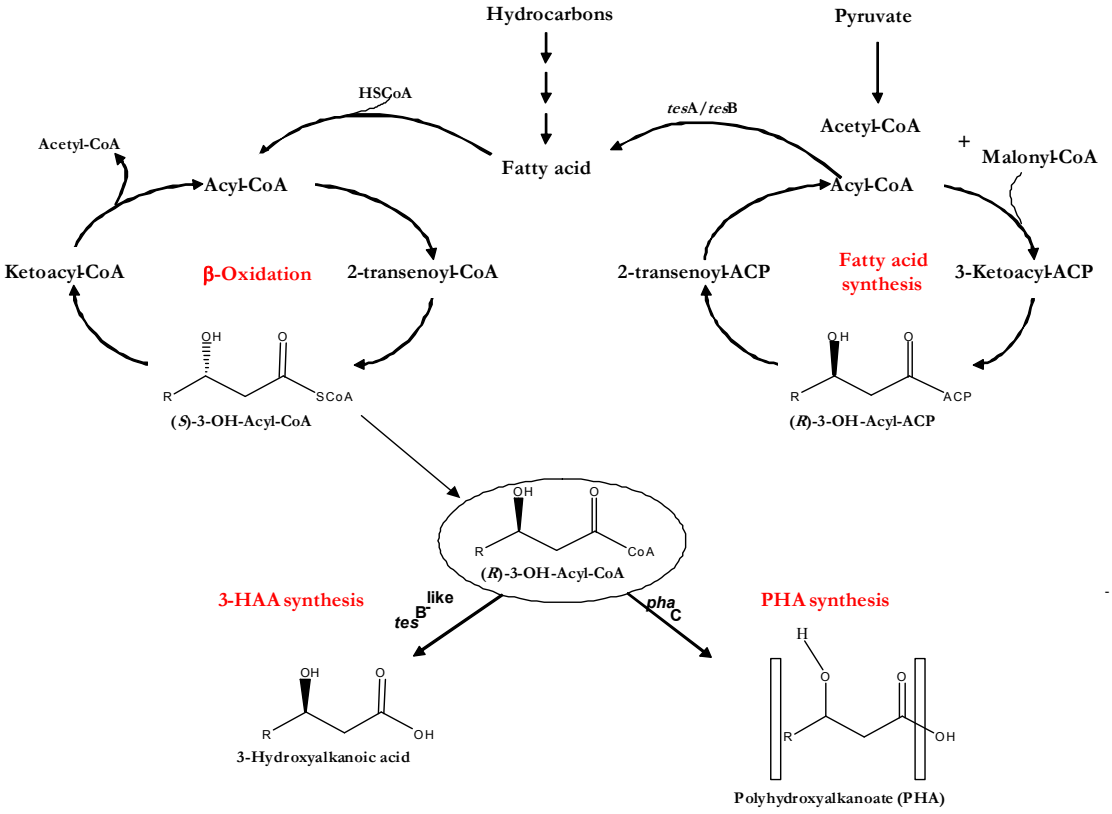


FIGURE 13. Probable pathway of PHA biosynthesis of A. borkumensis SK2 grown on hydrocarbons/pyruvate (Modified version of Klinke et al., 1999). Hydrocarbons are degraded via terminal oxidation through the sequential action of a monooxygenase, an alcohol dehydrogenase, and an aldehyde dehydrogenase to produce free fatty acids, which are then activated by acyl-CoA synthase and subjected to ß- oxidation[30]. The (S)-3-OH-acyl-CoAs produced by ß-oxidation are isomerised into (R)-3-OH-acyl-CoAs by the action of an isomerase. Pyruvate enters fatty acid biosynthesis in form of acetyl-CoA. Acyl-ACP produced in fatty acid biosynthesis is converted to free fatty acids by the action of TesA and TesB, which are then activated by acyl-CoA synthase and enter the ß-oxidation cycle. (R)-3-OH-acyl-CoAs produced during ß-oxidation are converted to either 3-hydroxyalkanoic acids (3-HAA), through the action of TesB-like acyl-CoA thioesterase or polyhydroxyalkanoate (PHA), through the action of PhaC synthase. The mutation in the TesB-like acyl-CoA thioesterase would abolish production of 3-HAA and channel (R)-3-OH-acyl-CoAs into PHA.

While the above consideration of the potential functional role of the TesB-like protein inactivated in the C9 mutant provides a simple and plausible explanation for the hyper-production of PHA by the mutant, the most interesting property of this mutant, namely its excretion of PHA, may either be a direct consequence of the *tesB*-like mutation-induced hyper-production of PHA, or the mutation may exert additional pleiotropic effects on cell physiology and in particular the cells' membrane composition. We found evidence that the latter is clearly changed in the C9 mutant strain as compared to the wild type (data not shown), most likely resulting from the inactivation of the hydroxyacyl-CoA-specific thioesterase (encoded by the mutated tesB-like gene) which leads to rerouting of alkane-derived fatty-acid chains entirely towards PHA synthesis and thus away from glyco- and phopholipid biosynthesis. Electron microscopic visualisation of shadow-casted excreting C9 mutant cells revealed large indentations or holes in the mutant cells membrane surface (data not shown), whereas the parental strain SK2 does not exhibit this feature. Since C9 seems to be perfectly healthy under conditions of PHA production from alkanes (Figure 8), these indentations are unlikely to be perforations of the cell surface and indeed, inspection of the ultra-thin section images showed intact cell membranes in the dent areas. Since it is known that PHA granula are bounded by a membrane that separates the hydrophobic PHA from the hydrophilic cytoplasm (Lundgren et al., 1964), one possible explanation of the facts that (i) PHA accumulates intracellularly in granules, (ii) PHA is excreted by the C9 mutant to the extracellular milieu as free polymer, and (iii) the mutant exhibits large indentations on its surface, is that the mechanism of PHA excretion involves transport of PHA granules through the cell envelope, perhaps mediated by membrane fusion events, where degranulation takes place, in a process reminiscent of lysosomal fusion in eukaryotes. The observed differences, both in composition and abundance, of membrane phospho- and glyco-lipids of the SK2 parental strain and the C9 mutant, particularly in octadecane-grown cells (data not shown) may well account for corresponding differences in membrane structure and functions associated with PHA transmembrane transport, including possible membrane fusion events leading to excretion of the PHA by the mutant.

4.4 Biotechnological potential of *Alcanivorax*: PHA as raw material for bioplastics

Polyhydroxyalkanoates (PHA), which are produced in form of intracellular granules by many microorganisms as carbon reserves during times of carbon surfeit (Kadouri et al., 2005), have long been explored as renewable resources for biodegradable thermoplastics and biopolymers (Anderson and Dawes, 1990; Steinbüchel, 1991). Currently, other potential applications are being assessed, including environmental and medical applications, such as the use of PHAs in wastewater denitrification bioreactors, in which they serve as both the source of reducing equivalents for denitrification and as the matrix for biofilm formation (Hiraishi and Khan, 2003), and as biocompatible materials for implants, scaffolds for tissue engineering, and drug carriers (Rivard et al., 1996; Nebe et al., 2001).

Despite their obvious advantages, PHA-based bioplastics are, because of their high production costs, not economically competitive with classical plastics and polymers from non-renewable petrochemicals. However, sustainability policies demand that sooner or later the switch be made to environmentally-friendly, biocompatible materials from renewable resources, so it is essential to develop new production systems that are more economical. A major component of the high production cost of PHA is its recovery from bacterial cells, in which it is stored in the form of PHA granula, through the use of large amounts of chemical reagents and/or enzymes. Approximately 20 parts of solvent are used to extract 1 part of polymer which, in addition to the cost and associated environmental disadvantage of large scale solvent usage, results in a significant reduction in molecular weight of the polymer, rendering it less useful for a number of industrial applications (Lee, 1996).

The novel *tesB*-like mutant strain of *Alcanivorax borkumensis*, isolated and described in this study, which hyper-produces PHA and excretes it into the medium constitutes the starting point of a new, economic and environmentally friendly developmental trajectory for the production of PHA. It should also be emphasized that the PHA hyper-production phenotype of the *tesB*-like mutant, a 160x increase in PHA production over that of the parental SK2 strain, though impressive and of considerable practical relevance, is simply the initial phenotype of the mutant, which can without doubt be improved significantly by normal process optimization practices.

4.5 Outlook

The present study has revealed many genes involved in adaptation of *Alcanivorax* SK2 to different environmental conditions characteristic for marine habitats, i.e. low temperature, salinity, UV radiation. Among them are several transcriptional regulators and sensor proteins mediating signal transduction, yet their target regulons have not been studied further. Therefore, in order to get a more comprehensive overview of the genes regulated by these transcriptional regulators functional genome analysis of respective mutants should be undertaken, using transcriptomic and/or proteomic approaches. The present study has also identified many genes involved in alkane metabolism in *Alcanivorax*. Among them is a putative monooxygenase encoded by ABO_0190, whose role in alkane oxidation is not yet clear, and it should thus be desirable to analyze the exact function of this particular monooxygenase in more detail. Moreover, all of the gene clusters involved in terminal oxidation of alkanes are preceded by different transcriptional regulators, yet the regulation of alkane degradation in *Alcanivorax* is not at all fully understood, in some instances even giving contradictory results. Here again, in order to understand how the complex system of transcriptional regulation of alkane terminal oxidation in *Alcanivorax* functions, the comprehensive identification of the regulons of all these regulators directly preceding operons of relevance for alkane degradation and thus likely to be involved in their regulation, would be of particular interest. In addition to regulatory proteins, a number of hypothetical proteins of unknown function, but found to be up-regulated on alkanes, should be further studied by characterizing these proteins at the biochemical level, e.g. with respect to potential structural or enzymatic features, eventual substrate specificities etc, as to understand how their function is related to alkane metabolism in *Alcanivorax*. Finally, this present work describes a novel phenotype of overproduction and excretion of PHA by *Alcanivorax*, as a consequence of a transposon mutation in a tesB-like gene of *Alcanivorax*. Since homologues to this gene are also present in some other PHA-producing gram-negative bacteria, like *Idiomarina loihiensis*, *Acinetobacter*, and *Pseudomonas putida*, corresponding tesB-like mutants of these strains might be other good or even better candidates for overproduction and excretion of the industrially highly interesting PHA, a raw material for bioplastics.

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- 1996-1997 – Mikrobieller Abbau schwefelhaltiger Bestandteile von Rohöl. Studienprojekt, Universität Kasan.
- 1995-1996 – Isolierung und Charakterisierung von aeroben Mikroorganismen, die an der Biodegradation von Dibenzothiophen mitwirken. Studienprojekt, Universität Kasan.

SPRACHKENNTNISSE

Russisch (Muttersprache), English (fliessend), Französisch (fortgeschritten), German (fortgeschritten).

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Tagungsbeiträge

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